FORM PTO-1390 (REV 5-93)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		2055MC/48896
		U S APPLICATION NO (if known, see 37 CFR 1 5)
		09/554414

INTERNATIONAL APPLICATION NO. PCT/CA98/01059	INTERNATIONAL FILING DATE 12 November 1998	PRIORITY DATE CLAIMED 12 November 1997

TITLE OF INVENTION
DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

APPLICANT(S) FOR DO/EO/US
Moshe SZYF; Sanjoy BHATTACHARYA; Shyam RAMCHANDANI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371

3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2)).

    a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).

    b. [X] has been transmitted by the International Bureau

    c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

    a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).

    b. [ ] have been transmitted by the International Bureau.

    c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.

    d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **UNEXECUTED**

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Item 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.

    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information:
    Form PCT/IB/308
    Copy of International Preliminary Examination Report
    Computer Readable Form of Sequence Listing
    Statement under 37 CFR 1.821 (f)
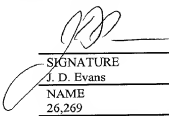   Executed Small Entity Status Declaration

U.S APPLICATION NO (if known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER	
09/554414	PCT/CA98/01059	2055MC/48896	
		CALCULATIONS	PTO USE ONLY

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO $840.00
International preliminary examination fee paid to USPTO (37 CFR 1.482) ... $670.00
No international preliminary examination fee paid to USPTO (37 CFR 1.482)

but international search fee paid to USPTO (37 CFR 1.445(a)(2) $760.00
Neither international preliminary examination fee (37 CFR 1.482) nor

international search fee (37CFR 1.445(a)(2) paid to USPTO .... $ 970.00
International preliminary examination fee paid to USPTO (37 CFR 1.482)

and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	31 -20=	11	X $18.00	$ 198.00	
Independent Claims	4 - 3=	1	X $78.00	$ 78.00	
Multiple dependent claims(s) (if applicable)			+ $260.00	$	

TOTAL OF ABOVE CALCULATIONS =		$1,246.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).		$	
SUBTOTAL =		$ 623.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		$ +	
TOTAL NATIONAL FEE =		$ 623.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28,3.31). $40.00 per property +		$	
TOTAL FEE ENCLOSED =		$ 623.00	
		Amount to be:	$
		refunded	
		charged	$

a. [X] A check in the amount of $ 623.00 for the filing fee is enclosed
b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A

duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees, which may be required, or credit any overpayment to

Deposit Account No. _05-1323_ . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or

(b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Evenson, McKeown, Edwards & Lenahan, P.L.L.C.
1200 G Street, N.W., Suite 700
Washington, D.C. 20005
Tel. No. (202) 628-8800
Fax No. (202) 628-8844

SIGNATURE
J. D. Evans
NAME
26,269
REGISTRATION NUMBER
May 12, 2000

U.S. APPLICATION NO. (if known, see 37 CFR 1.5 **097554414**	INTERNATIONAL APPLICATION NO PCT/CA98/01059	ATTORNEY'S DOCKET NUMBER 2055MC/48896	

		CALCULATIONS	PTO USE ONLY
17. [X] The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO .... $840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ... $670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2) .... $760.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2) paid to USPTO .... $ 970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00			

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	

Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	31 -20=	11	X $18.00	$ 198.00	
Independent Claims	4 - 3=	1	X $78.00	$ 78.00	
Multiple dependent claims(s) (if applicable)			+ $260.00	$	

TOTAL OF ABOVE CALCULATIONS =	$1,246.00	

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).	$	

SUBTOTAL =	$ 623.00	

Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	

TOTAL NATIONAL FEE =	$ 623.00	

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28,3.31). $40.00 per property +	$	

TOTAL FEE ENCLOSED =	$ 623.00	

	Amount to be:	$
	refunded	
	charged	$

a. [X] A check in the amount of $ 623.00 for the filing fee is enclosed
b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A

  duplicate copy of this sheet is enclosed.
c. [X] The Commissioner is hereby authorized to charge any additional fees, which may be required, or credit any overpayment to

  Deposit Account No. 05-1323 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or

(b)) must be filed and granted to restore the application to pending status.

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Evenson, McKeown, Edwards & Lenahan, P.L.L.C.
1200 G Street, N.W., Suite 700
Washington, D.C. 20005
Tel. No. (202) 628-8800
Fax No. (202) 628-8844

SIGNATURE
J. D. Evans
NAME
26,269
REGISTRATION NUMBER
May 12, 2000

Attorney Docket: 2055MC/48896
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: MOSHE SZYF ET AL.

Serial No.: UNASSIGNED   Group Art Unit: UNASSIGNED

Filed: MAY 12, 2000   Examiner: UNASSIGNED

Corresponding to PCT/CA98/01059

November 12, 1998

Title: DNA DEMETHYLASE, THERAPEUTIC AND
        DIAGNOSTIC USES THEREOF

PRELIMINARY AMENDMENT

Box PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

     Please enter the following amendments to the claims, prior

to the examination of the application during the U.S. National

Phase.

IN THE CLAIMS:

     Claim 4, line 2, change "claims" to --claim-- and delete

"or 3".

     Claim 10, line 2, change "claims" to --claim-- and delete

"or 3".

Claim 11, line 2, change "claims" to --claim-- and delete "or 3".

Claim 15, line 1, delete "or 3".

Claim 16, line 1, delete "or 3".

Claim 19, line 2, delete "or 2".

Claim 23, line 1, change "one of claims" to --claim-- and delete "or 22".

Claim 27, line 1, delete "or its cDNA of";
line 2, delete "claim 2".

Claim 28, line 1, delete "or its cDNA";
line 2, delete "of claim 2".

Please add the following new claims:
--   30.   Use of the cDNA of claim 2, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

31. Use of the cDNA of claim 2, for inhibiting methylation in cancel cells using vector mediated gene therapy. --
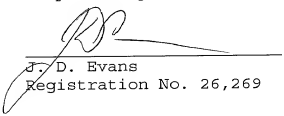
R E M A R K S

Entry of the amendments to the claims before examination of the application is respectfully requested. These claims have been amended to remove multiple dependencies.

If there are any questions regarding this Preliminary Amendment or this application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

Respectfully submitted,

May 12, 2000

J. D. Evans
Registration No. 26,269

EVENSON, McKEOWN, EDWARDS
   & LENAHAN, P.L.L.C.
1200 G Street, N.W., Suite 700
Washington, DC 20005
Telephone No.: (202) 628-8800
Facsimile No.: (202) 628-8844

Applicant or Patentee: _____ Moshe Szyf et al. _____

Serial or Patent No.: _____ Atty. Dkt. No.: _____ 1770-183"US-FC _____

Filed or Issued: _____

For: __ DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF _____

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) AND 1.27 (d)] - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION _____ McGILL UNIVERSITY _____

ADDRESS OF ORGANIZATION _____ 845 Sherbrooke Street West _____

_____ Montréal, Québec, Canada H3A 2T5 _____

TYPE OF ORGANIZATION

(X) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

( ) TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) AND 501(c)(3)]

( ) NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED
STATES OF AMERICA

(NAME OF STATE _____)

(CITATION OF STATUTE _____)

( ) WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC
501(a) AND 501 (c)(3)] IF LOCATED IN THE UNITED STATES OF AMERICA

( ) WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF
STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES
OF AMERICA

(NAME OF STATE _____)

(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37
CFR 1.9 (e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to
the invention ___ DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF __ by inventor(s)
___ Moshe Szyf; Sanjoy Bhattacharya; and Shyam Ramchandani __ described in

( ) the specification filed herewith

( ) application serial no. _____ , filed _____

( ) patent no. _____ , issued _____ .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-
identified invention.
If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed
below* and no rights to the invention are held by any person, other than the inventor who could not qualify as an independent inventor under 37 CFR
1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).
NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their
status as small entities. [37 CFR 1.27]

NAME _____

ADDRESS _____

( ) INDIVIDUAL   ( ) SMALL BUSINESS CONCERN   ( ) NONPROFIT ORGANIZATION
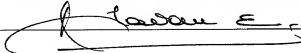
NAME _____

ADDRESS _____

( ) INDIVIDUAL   ( ) SMALL BUSINESS CONCERN   ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status
prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no
longer appropriate. [37 CFR 1 28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to
be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or
imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of
the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING _____ Alex Navarre _____

TITLE IN ORGANIZATION _____ Director – Office of Technology Transfer _____

ADDRESS OF PERSON SIGNING____ 845 Sherbrooke Street West, Quebec, Canada H3A 2T5 __

SIGNATURE _____ DATE _09/05/2000_

Attorney Docket: 2055MC/48896
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: MOSHE SZYF ET AL.

Serial No.: UNASSIGNED   Group Art Unit: UNASSIGNED

Filed: MAY 12, 2000   Examiner: UNASSIGNED

Corresponding to PCT/CA98/01059

November 12, 1998

Title: DNA DEMETHYLASE, THERAPEUTIC AND
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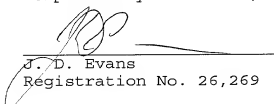
STATEMENT UNDER 37 C.F.R. 1.821(f)

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

        The undersigned hereby states that the contents of the
paper copy of the sequence listing in the accompanying U.S.
National Stage patent application and the accompanying computer
readable copy of the sequence listing are the same.

                              Respectfully submitted,

May 12, 2000

                              J. D. Evans
                              Registration No. 26,269

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JDE:dcb

DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

BACKGROUND OF THE INVENTION

5   (a) Field of the Invention

The invention relates to a novel enzyme, DNA demethylase, therapeutic and diagnostic uses thereof.

(b) Description of Prior Art

Many lines of evidence have established that
10   modification of cytosine moieties residing in the dinu-
cleotide sequence CpG in vertebrate genomes is involved
in regulating a number of genome functions such as
parental imprinting, X-inactivation, suppression of
methylation of ectopic genes and differential gene
15   expression (Szyf, M. (1996) Pharmacol. Ther. 70, 1-37).
DNA methylation performs its function by differentially
marking genes because the distribution of methylated
CpGs is tissue- and site- specific forming a pattern of
methylation (Szyf, M. (1996) Pharmacol. Ther. 70, 1-
20   37). It is clear that the pattern of methylation is
fashioned by a sequence of methylation and demethyla-
tion events (Brandeis, M. et al. (1993) Bioassays 15,
709-713) during development and is maintained in the
fully differentiated cell (Razin, A. et al. (1980) Sci-
25   ence 210, 604-610). While it was originally suggested
that DNA demethylation is accomplished by a passive
loss of methyl groups during replication (Razin, A. et
al. (1980) Science 210, 604-610), it is now clear that
an active process of demethylation occurs in embryonal
30   cells (Frank, D. et al. (1991) Nature 351, 239-241), in
differentiating cell lines (Razin, A. et al. (1986)
Proc. Natl. Acad. Sci. USA 83, 2827-2831; Szyf, M. et
al. (1985) Proc. Natl. Acad. Sci. USA 82, 8090-8094)
and in response to estrogen treatment (Saluz, H.P. et
35   al. (1986) Proc. Natl. Acad. Sci. USA 83, 7167-7171).
Two modes of demethylation have been documented: site

specific demethylation that coincides in many instances
with onset of gene expression of specific genes and a
general genome wide demethylation that occurs during
early development *in vivo* during cellular differentia-
tion and in cancer cells (Feinberg, A.P. et al. (1983)
Nature *301*, 89-92; Razin, A. et al. (1986) Proc. Natl.
Acad. Sci. USA *83*, 2827-2831). The global demethyla-
tion is consistent with the hypothesis that a general
demethylase activity which is activated at specific
points in development or oncogenesis exists. It has
been hypothesized that one mechanism regulating the
pattern of methylation is the control of expression of
methyltransferase (Szyf, M. (1991) Biochem. Cell Biol.
69, 764-767) and demethylase activities (Szyf, M.(1994)
Trends Pharmacol. Sci. 7, 233-238). Although exten-
sive information has been obtained on the enzymatic
activity responsible for methylation and the regulation
of its expression in the last two decades (Szyf, M.
(1996) Pharmacol. Ther. *70*, 1-37), the identity of the
demethylase has remained a mystery. It is clear how-
ever that to fully understand how patterns of methyla-
tion are formed and maintained and to determine their
role in development, physiology and oncogenesis, one
has to identify the demethylase enzyme(s). Two main
difficulties have inhibited the identification of this
enzyme. First, it is believed that demethylation of a
methylated cytosine is chemically highly unlikely since
it involves breaking a very stable C-C bond. Second,
demethylation occurs at very defined stages in develop-
ment (Brandeis, M. et al. (1993) Bioassays *15*, 709-713)
and identifying an adequate tissue source for this
enzyme is critical.

Whereas no *bona fide* demethylase has been iden-
tified to date, alternative biochemical mechanisms
involving exchange of methylated cytosines with non-

methylated cytosines have been described. One previ-
ously proposed mechanism is removal of the methylated
base by a glycosylase and its replacement with a non-
methylated nucleotide utilizing an "excision-repair"
mechanism (Razin, A. et al. (1986) Proc. Natl. Acad.
Sci. USA *83*, 2827-2831). Glycosylase activities that
can remove methylated cytosines from DNA have been dem-
onstrated by Vairapandi and Duker (Vairapandi, M. et
al. (1993) *Nucl. Acids Res. 21*, 5323-5327) and more
recently by Jost (Jost, J. P. et al. (1995) J. Biol.
Chem. *270*, 9734-9739).   However it is not clear
whether this activity is responsible for the general
demethylation observed in cellular differentiation.
The fact that the activity identified by Jost acts spe-
cifically on hemimethylated sequences (which is not the
natural substrate in most cases) and can remove thymi-
dines as well as 5-methylcytosines, supports a repair
function for this glycosylase-demethylase (Jost, J. P.
et al. (1995) J. Biol. Chem. *270*, 9734-9739).   An
alternative mechanism involving a RNA dependent activ-
ity has been recently described by Weiss et al. (Weiss
et al., 1996). This proteinase-insensitive RNA depend-
ent activity has been shown to catalyze the excision
and replacement of a methylated CpG dinucleotide with a
nonmethylated CpG dinucleotide that is contained in a
DNA-RNA hybrid molecule (Weiss, A. et al. (1996) Cell
87, 709-718). This activity which was identified in
differentiating cells in culture was proposed to be
involved in demethylation during development.   These
previous findings demonstrate that the common accepted
model in the filed has been that a *bona fide* demethy-
lase does not exist.

It has been previously proposed that the exten-
sive hypomethylation observed in cancer cells might be
a consequence of activation of demethylase activity by

- 4 -

oncogenic pathways (Szyf, M.(1994) Trends Pharmacol.
Sci. 7, 233-238; Szyf, M. et al. (1995) J. Biol. Chem.
270, 12690-12696). In accordance with this hypothesis
we have shown that ectopic expression of v-Ha-ras had
5   induced demethylation activity in the cells (Szyf, M.
et al. (1995) J. Biol. Chem. 270, 12690-12696). Using
an assay that directly measures the conversion of 3'^{32}P
labeled methyl dCMP (mdCMP) into dCMP, we have shown
that nuclear extracts prepared from P19-Ras transfec-
10 tants bear high levels of demethylase activity (Szyf,
M. et al. (1995) J. Biol. Chem. 270, 12690-12696).
Building on this observation, we hypothesized that can-
cer cell lines were a good source for demethylase.
However, it is not evident that Ras expression in p19
15 cells does reflect the situation in cancer cells. P19
is an embryonic cell and expression of Ras might be
differentiating them.
        It would be highly desirable to be provided with
a bona fide DNA demethylase (DNA dMTase) to alter
20 developmental programs for therapeutic and biological
use.

SUMMARY OF THE INVENTION
        In accordance with the present invention, we
25 demonstrate the purification of a bona fide DNA demeth-
ylase (DNA dMTase) from a human lung cancer cell line
A549, determine its kinetic parameters and substrate
specificity. The DNA dMTase activity identified in
this study converts methyl-dCMP (mdCMP) residing in the
30 dinucleotide sequence mdCpG into dCMP whereas the
methyl group is released as a volatile residue which
was identified to be methanol. The activity is puri-
fied away from any trace amounts of dCTP, is insensi-
tive to the DNA polymerase inhibitor ddCTP, is not
35 affected by the presence of methyl dCTP (mdCTP) in the

reaction and does not exhibit exonuclease or glyco-
sylase activities.   The identification of this new
enzyme points out to new directions in our understand-
ing of how DNA methylation patterns are formed and
5   altered.

        One aim of the present invention is to provide a
bona fide DNA demethylase (DNA dMTase).

        In accordance with the present invention there
is provided a DNA demethylase enzyme having about
10   40 KDa, and wherein the DNA demethylase enzyme is over-
expressed in cancer cells and not in normal cells.

        In accordance with the present invention there
is provided a cDNA encoding human demethylase which
comprises a sequence set forth in SEQ ID NO:1.

15   In accordance with the present invention there
is provided two mouse cDNAs homologous to the human
cDNA, wherein the cDNA encoding mouse demethylase hav-
ing a sequence set forth in SEQ ID NOS:5-7.

        In accordance with the present invention there
20   is provided a different human cDNA which encodes a pro-
tein homologous to the human demethylase having a
sequence set forth in SEQ ID NO:3.

        In accordance with the present invention there
is provided the use of the expression of demethylase
25   cDNAs to alter DNA methylation patterns of DNA *in vitro*
in cells or *in vivo* in humans, animals and in plants.

        The demethylase cDNAs expression may be under
the direction of mammalian promoters, such as CMV.

        The demethylase cDNAs expression may be under
30   plant specific promoters to alter methylation in plants
and to allow for altering states of development of
plants and expression of foreign genes in plants.

        The demethylase cDNAs expression may be in the
antisense orientation to inhibit demethylase in cancer
35   cells for therapeutic processes.

- 6 -

The expression of demethylase cDNA in mammalian cells may be to alter their differentiation state and to generate stem cells for therapeutics, cells for animal cloning and to improve expression of foreign genes.

5   In accordance with the present invention there is provided the use of the expression of demethylase cDNAs in bacterial or insect cells for production of large amounts of demethylase.

In accordance with the present invention there 10   is provided the use of the expression of demethylase cDNAs for the production of protein in vertebrate, insect or bacterial or plant cells, such as antibodies against demethylase.

In accordance with the present invention there 15   is provided the use of the sequence of demethylase cDNAs as a template to design antisense oligonucleotides and ribozymes.

In accordance with the present invention there is provided the use of the predicted peptide sequence 20   of demethylase cDNAs to produce polyclonal or monoclonal antibodies against demethylase.

In accordance with the present invention there is provided the use of expression of cDNAs in two hybrid systems in yeast to identify proteins interact- 25   ing with demethylase for diagnostic and therapeutic purposes.

In accordance with the present invention there is provided the use of expression of cDNAs in bacterial, vertebrate or insect cells to produce large 30   amounts of demethylase for obtaining a x-ray crystal structure and for high throughput screening of demethylase inhibitors for therapeutics and biotechnology.

In accordance with the present invention there is provided a volatile assay for high throughput

screening of demethylase inhibitors as therapeutics and
anticancer agents which comprises the steps of:

    a)   using transcribed and translated demethylase
        cDNAs *in vitro* to convert methyl-cytosine pres-
5   ent in methylated DNA samples to cytosine pres-
        ent in DNA and volatilize methyl group;

    b)   determining the absence or minute amount of
        volatilize methyl group as an indication of an
        active demethylase inhibitor.

10   In accordance with the present invention there
is provided a volatile assay for the diagnostics of
cancer in a patient sample which comprises the steps
of:

    a)   determining demethylase activity in patient sam-
15   ples by assaying conversion of methyl-cytosine
        present in methylated DNA to cytosine present in
        DNA and its volatilization as methyl groups
        released as methanol;

    b)   determining the presence or minute amount of
20   volatilized methyl released as methanol groups
        as an indication of cancer in the patient sam-
        ple.

      In accordance with the present invention there
is provided the use of an antagonist or inhibitor of
25 DNA demethylase for the manufacture of a medicament for
cancer treatment, for restoring an aberrant methylation
pattern in a patient DNA, or for changing a methylation
pattern in a patient DNA.

      Such an antagonist is a double stranded oligonu-
30 cleotide that inhibits demethylase at a Ki of 50nM,
such as $\left[\begin{matrix} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{matrix} \right]_n$.

      The inhibitors include, without limitation an
anti-DNA demethylase antibody, an antisense of DNA
35 demethylase or a small molecule such as any derivative
of imidazole.

            The change of the methylation pattern may acti-
    vate a silent gene. Such an activation of a silent gene
    permits the correction of genetic defect such as found
    for β-thalassemia or sickle cell anemia.

5   The DNA demethylase of the present invention may
    be used to remove methyl groups on DNA *in vitro* such as
    needed for cloning DNA.

            The DNA demethylase of the present invention or
    its cDNAs may be used, for changing the state of dif-
10 ferentiation of a cell to allow gene therapy, stem cell
    selection or cell cloning.

            The DNA demethylase of the present invention or
    its cDNAs may be used, for inhibiting methylation in
    cancer cells using vector mediated gene therapy.

15   In accordance with the present invention there
    is provided an assay for the diagnostic of cancer in a
    patient, which comprises determining the level of
    expression of DNA demethylase by either RT-PCT, ELISA
    or volatilization assay of the present invention in a
20 sample from the patient, wherein overexpression of the
    DNA demethylase is indicative of cancer cells.

    BRIEF DESCRIPTION OF THE DRAWINGS

            Figs. 1A to 1B illustrate the purification of
25 demethylase (DNA dMTase) from human A549 cells;

            Figs. 2A and 2C illustrate that DNA dMTase is a
    protein inhibited by RNA and not by ddCTP, mdCTP;

            Figs. 2B and 2D illustrate the kinetics of DNA
    dMTase activity;

30   Figs. 3A to 3C illustrate the product of DNA
    dMTase activity is cytosine and it exhibits no exonu-
    clease or glycosylase activity;

            Figs. 4A-4C illustrate the demethylation reac-
    tion releases methanol as a volatile residue;

Fig. 4D illustrates the transfer of a proton from water to regenerate cytosine;

Figs. 4E-4F illustrate that the volatile product is methanol;

Fig. 5 illustrates the suggested demethylation reaction;

Figs. 6A-6D illustrate the substrate Specificity of DNA dMTase;

Figs. 7A-7D illustrate chromatographic isolation of dMTase from human A549 cells;

Figs. 8A-8B illustrate the alignment between the MDB domain of MeCP2 and demethylase and the predicted amino acid sequence of human demethylase;

Fig. 8C illustrates the mRNA encoded by demethylase;

Figs. 9A-9F illustrate the cDNA and their predicted amino acid of demethylases and homologues of the present invention (SEQ ID NOS:1-8);

Figs. 10A-B illustrate a mammalian expression vector of dMTase and in vitro translated dMTase polypeptide;

Fig. 10C illustrates that in vitro translated DNA dMTase releases volatile methyl residues from methylated DNA;

Fig. 10D illustrates that in vitro translated DNA dMTase transform methylated cytosines to cytosines;

Fig. 11A illustrates that transiently transfected demethylase releases volatile residues from methylated DNA;

Fig. 11B illustrates the polypeptide expressed from transiently transfected demethylase;

Figs. 11C-11E illustrate that transiently transfected demethylase transforms methylated cytosines to cytosines in a protein dependent manner;

Fig. 11F illustrates that the transformation of methylated cytosine to cytosine by transiently transfected demethylase depends on the concentration of substrate;

5   Fig. 12A illustrates that transiently transfected demethylase catalyzes the transfer of a proton from tritiated water to regenerate cytosine;

Fig. 12B illustrates that the cloned demethylase releases methanol from methylated DNA;

10   Figs. 13A-13C illustrate that the cancer cells express demethylase activity whereas normal cells do not;

Fig. 13D illustrates that demethylase mRNA is highly express in cancer cells;

15   Fig. 14A illustrates demethylase bacterial retroviral and mammalian expression vector;

Fig. 14B illustrates inhibition of demethylase activity by a specific inhibitor;

Fig. 14C illustrates inhibition of tumorigenesis
20   in vitro by an inhibition of demethylase;

Fig. 15 illustrates inhibition of tumorigenesis in cell culture by induced expression of demethylase antisense vector;

Fig. 16 illustrates the inhibition of demethy
25   lase by a small molecule inhibitor imidazole; and

Fig. 17 illustrates a model for the inhibition of cancer growth by an inhibition of demethylase.

DETAILED DESCRIPTION OF THE INVENTION

30   The pattern of methylation is fashioned during development by a sequence of methylation and demethylation events. The identity of the demethylase has remained a mystery and alternative biochemical activities have been shown to demethylate DNA but no activity
35   that can truly remove methyl groups from DNA has been

shown to date. Utilizing human lung carcinoma cells as
a source for demethylase activity we demonstrate that
mammalian cells bear a *bona fide* DNA demethylase (DNA
dMTase) activity. DNA dMTase transforms methyl-C to C
by catalyzing replacement of the methyl group on the 5
position of C with a hydrogen derived from water. DNA
dMTase demethylates both fully methylated and hemimeth-
ylated DNA, shows dinucleotide specificity and can
demethylate mdCpdG sites in different sequence con-
texts.   This enzyme is different from previously
described demethylation activities: it is proteinase
sensitive, activated by RNase and releases different
products.

          DNA dMTase is a novel enzyme showing a new and
unexpected activity that has not been previously
described in any organism. The finding of a *bona fide*
demethylase, points out new directions in our under-
standing of the biological role of DNA methylation.

          In spite of the fact that it was previously
shown that Ras expression in p19 cells can induce
demethylation activity. It was not clear whether this
demethylation activity is indeed a *bona fide* demethy-
lase. One would predict that demethylase is present in
embryonal cells. It was surprising to see that demeth-
ylation activity is present in cancer cells. The find-
ing of high levels of demethylase in A549 cells is
indeed an unexpected discovery.

          In accordance with the present invention, it is
shown and demonstrated that demethylation occurs by
removal of a methyl group from methylated cytosine in
DNA, that a hydrogen from water replaces the methyl
group at the 5' position, that the resulting methyl
group reacts with the remaining hydroxyl from water to
generate methanol which volatilizes (Fig. 4E-F). Thus,

bona fide demethylation of DNA involves the following
reaction:

$$CH_3\text{-cytosine-(DNA)} + H\text{-OH} \xrightarrow{\text{demethylase}} H\text{-cytosine} + CH_3\text{-OH}$$
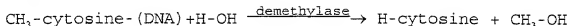
5   The cDNA cloned in accordance with the present
invention is the demethylase since it can convert
methyl-cytosines in DNA to cytosines and volatilize the
methyl groups on DNA when transcribed and translated *in
vitro* which are released as methanol. This is a novel
10 cDNA encoding a biochemical activity that has been not
described before.

In accordance with the present invention, there is
shown a model for the inhibition of cancer growth by an
inhibition of demethylase (Fig. 17).

15

EXPERIMENTAL PROCEDURES
Cell Culture
        A549 Lung Carcinoma cells (ATCC: CCL 185) were
grown in Dulbecco's modified Eagle's medium (with low
20 glucose) supplemented with 10% fetal calf serum, 2 mM
glutamine, 10 U/ml cifrofloxacin.   Human Skin Fibro-
blasts #72-213A MRHF were obtained from BioWhittaker,
Bethesda and were grown in Dulbecco's modified Eagle's
medium supplement with 2% fetal calf serum, 2 mM gluta-
25 mine. H446 Lung carcinoma cells (ATCC: HTB 171) was
grown in RPMI 1640 medium with 5% fetal calf serum.
Preparation of nuclear extract
        Nuclear extracts were prepared from A549 cul-
tures at near confluence as previously described (Szyf
30 et al., 1991; Szyf et al.,1995). The cells were tryp-
sinized, collected and washed with phosphate-buffered
saline and suspended in buffer A (10 mM Tris, pH 8.0,
1.5 mM MgCl$_2$, 5mM KCl, 0.5% NP-40) at the concentration
of 10^8 cells per ml for 10 min. at 4°C. Nuclei were
35 collected by centrifugation of the suspension at 1000 g

for 10 minutes. The nuclear pellet was resuspended in
buffer A (400 μl) and collected as described in the
experimental procedures.   A nuclear extract was pre-
pared from the pelleted nuclei by suspending them in
5   buffer B (20 mM Tris, pH 8.0, 25% glycerol, 0.2 mM EDTA
and 0.4 mM NaCl) at the concentration of $3.3x10^8$ nuclei
per ml and incubating the suspension for 15 min. at
4°C.   The nuclear extract was separated from the
nuclear pellet by centrifugation at 10,000g for 30 min-
10   utes. Nuclear extract was stored in -80°C for at least
two months without loss of activity.

Chromatography on DEAE-Sephadex

A freshly prepared nuclear extract (1 ml , 1.1
mg) was passed through a Microcon™ 100 spin column, the
15   retainant was diluted to a conductivity equivalent to
0.2 M NaCl in buffer L and applied onto a DEAE-Sephadex
column (Pharmacia) (1.0 x 5 cm) that was preequili-
brated with buffer L (10 mM Tris-HCl, pH 7.5, 10 mM
$MgCl_2$) containing 0.2 M NaCl at a flow rate of 1
20   ml/min.   The column was then washed with 15 ml of the
starting buffer (buffer L + 0.2 M NaCl) and proteins
were eluted with 5 ml of a linear gradient of NaCl
(0.2-5.0 M).   0.8 ml fractions were collected and
assayed   for   demethylase   activity   after   desalting
25   through a Microcon™ 10 spin column (Amicon) and resus-
pension of the retainant in 0.8 ml buffer L.   DNA
demethylase eluted between 2-5.0 M NaCl.

Chromatography on S-Sepharose

Active   DEAE-Sepharose   column   fractions   were
30   pooled, adjusted to 0.1 M NaCl by dilution and loaded
onto an S-sepharose column (Pharmacia) (1.0 x5 cm)
which had been preequilibrated with buffer L containing
0.2 M NaCl at a flow rate of 1 ml/min.   Following wash-
ing of the column as described in experimental proce-
35   dures, the proteins were eluted with 5 ml of a linear

NaCl gradient (0.2-5.0M). 0.5 ml fractions were col-
lected and assayed for DNA demethylase activity after
desalting and concentrating to 0.2 ml using a Microcon™
10 spin column. DNA demethylase activity eluted around
5   5.0 M NaCl.

Chromatography on Q-Sepharose

Active fractions from S-sepharose column were
pooled, adjusted to 0.2 M NaCl by dilution and applied
onto a Q-sepharose (Pharmacia) column (1.0 x5 cm) which
10 had been equilibrated as described in the experimental
procedures at a flow rate of 1 ml/min. The column was
washed and the proteins were eluted with a linear NaCl
gradient (0.2- 5.0 M). Fractions (0.5 ml) were col-
lected, assayed for demethylase activity after desalt-
15 ing and concentrating to a final volume of 0.2 ml as
described in the experimental procedures. The demethy-
lase activity eluted around 4.8-5.0 M NaCl.

Gel-Exclusion Chromatography on DEAE-Sephacel

The pooled fractions of Q-sepharose column were
20 adjusted to 0.2 M NaCl, loaded onto a 2.0 x 2.0 cm
DEAE-Sephacel column (Pharmacia) and eluted with 10 ml
of buffer L containing 0.2 M NaCl. The fractions (0.8
ml) were collected and assayed after concentration to
about 180 μl with a Microcon™ 10 spin column for DNA
25 demethylase activity. The activity was detected at
fraction 4, which is very near the void volume
(~200kDa).

Assay of DNA demethylase activity

To directly assay DNA demethylase activity *in*
30 *vitro* two independent methods were applied.

(A) To assay the conversion of methyl-dCMP (mdCMP) to
dCMP we used a previously described method (Szyf et
al., 1995). Briefly, $\alpha^{32}P$ labeled, fully methylated
poly[mdC^{32}PdG]n substrate was prepared as follows. One
35 hundred ng of a double-stranded fully methylated

(mdCpdG) oligomer (Pharmacia) were denatured by boil-
ing, which was followed by partial annealing at room
temperature. The complementary strand was extended
with Klenow fragment (Boehringer Mannheim) using
5   methyl-5-dCTP (mdCTP, 0.1 mM) (Boehringer Mannheim) and
[α-^{32}P] GTP (100 μCi, 3000 Ci/mmol), and the unincorpo-
rated nucleotides were removed by chromatography
through a NAP-5 column (Pharmacia). The NAP-5 chroma-
tography was repeated to exclude minor contamination
10 with unincorporated nucleotides. As a control a non-
methylated poly[dC^{32}pdG]n substrate was similarly pre-
pared except that a nonmethylated dCpdG oligomer served
as a template and dCTP was used in the extension reac-
tion. The column fractions (30 μl), described in the
15 experimental procedures were incubated with 1 ng of
poly[mdC^{32}pdG]n substrate for 1 hour at 37°C in a
buffer L containing 25% glycerol (v/v) and 5 mM EDTA.
The reacted DNA as well as a nonmethylated
poly[dC^{32}pdG]n and methylated [mdC^{32}pdG]n nonreacted con-
20 trols were purified by phenol/chloroform extraction and
subjected to micrococcal nuclease digestion (100 μg at
10 μl) and calf spleen phosphodiesterase (2μg)
(Boehringer) (Pharmacia) to 3′ mononucleotides for 15
hours at 37°C. The digestion products were loaded onto
25 a thin layer chromatography plate (TLC) (Kodak, 13255
Cellulose), separated in a medium containing, 132 ml
Isobutyric acid:40 ml water: 4 ml ammonia solution,
autoradiographed and the intensity of the different
spots was determined using a phosphorimager (Fuji, BAS
30 2000). ^{32}P labeled substrates and tritium labeled sub-
strates were phosphoimaged using BAS 2000 plate and
BAS-TR2040 phosphorimager plate respectively.
(B) The second method determined removal of methylated
residues from methylated DNA by measuring disappearance
35 of ^{3}H-CH$_{3}$ or ^{14}C-CH$_{3}$ from the reaction mixture. 100 ng

of poly [dCdG]n double stranded DNA was methylated
using SssI methylase (New England Biolabs) and an
excess of [^{3}H-methyl AdoMet (80 Ci/mmol; New England
Nuclear)]. The tritiated methyl group containing DNA

5   was purified from labeled AdoMet using NAP-5 column
chromatography. All column purified fractions of DNA
demethylase were assayed using the tritiated substrate.
In a typical assay, 1 ng of DNA was incubated (at a
specific activity of 4 x10^{6}dpm/mg)with 30 μl of column

10   fraction for one hour at 37°C in buffer L. To deter-
mine the number of methyl groups remaining in the DNA
following incubation with the different fractions, 250
μl of water were added and the mixture was incubated at
65°C for 5 minutes. One hundred μl of the reaction

15   mixture were withdrawn for liquid scintillation count-
ing. Controls received similar treatment except that
in place of a column fraction, an equal volume of
buffer L was added. The number of methyl groups that
were removed from the DNA by the different fractions

20   was determined by subtracting the remaining counts in
each of the fractions from the counts remaining in the
control. All tests were carried out in triplicates.
The results are presented as picomole methyl group
removed. One unit of DNA dMTase activity is defined

25   as: amount of enzyme that releases one picomole of
methyl group from methylated dCpdG substrate in one
hour at 37°C.
Methyl removal assay using double-labeled substrates
       To determine whether the methyl group leaves the

30   DNA and not any non-specific removal of tritium, we
prepared SK plasmid DNA containing a tritiated hydrogen
at the 6' position of cytosine and thymidine by growing
the plasmid harboring bacteria in the presence of deoxy
[6-^{3}H] Uridine (22 Ci/mmol; Amersham) (10μCi/ml). The

35   [6-^{3}H]-cytosine containing pBluescript SK(+) was puri-

fied according to standard protocols and was methylated
using an excess of [^{14}C-methyl] AdoMet (59 mCi/mmol;
Amersham) (10 μCi per 100 μl reaction)and SssI methy-
lase. The double labeled DNA substrate was purified

5   twice on a NAP-5 column.   15 μl of DNA dMTase were
incubated with 1 ng of double labeled DNA (specific
activity of 2000 dpm/ng) for 1 hour at 37°C.   Follow-
ing incubation, the remaining ^{14}C versus ^{3}H counts were
determined as described in the experimental procedures

10 by scintillation counting (Wallac).   The ^{14}C counts were
normalized against ^{3}H counts.   The controls received
similar treatment except that instead of DNA dMTase, an
equal amount of distilled water was added to them.

To determine the number of ^{3}H-CH$_3$ in the gaseous

15 phase, 1 ng of ^{3}H-CH$_3$ poly [dCpdG] DNA were incubated
with DNA dMTase overnight in a sealed tube (Pierce,
Illinois, USA). 0.8 ml of air were removed from the
tube using a gas tight syringe (Hamilton, Reno, Nevada)
and injected into a sealed gas tight scintillation vial

20 containing 10 ml OptiPhase scintillation fluid (Wallac,
UK) and counted. As a control the DNA was incubated
with an equal volume of buffer L and treated similarly.
Synthesis of other methylated dC dinucleotides

Poly [mdC^{32}pdA] and [mdC^{32}pdT] substrates were

25 prepared as follows. About 0.5 μg of 20 mer oligonu-
cleotides 5'(GG)103', 5'(GT)103' and 5'(GA)103' were
boiled and annealed at room temperature with oligonu-
cleotide 5'CCCCCC3', 5'CACACA3' and 5'CTCTCT3' respec-
tively. The complementary strand was extended with

30 Klenow fragment using m5dCTP (Boehringer Mannheim) and
either [α^{32}P] dATP (100μCi, 3000Ci/mmol) or [α^{32}P] dTTP
(100 μCi, 3000 Ci/mmol) respectively. The unincorpo-
rated nucleotides were removed by chromatography
through a NAP-5 column. Hemimethylated mdCpG substrate

35 was prepared in a similar manner except that a nonmeth-

ylated poly dCpdG substrate (Boehringer) was used as
template and m5dCTP and $[\alpha^{32}P]$dGTP were used for exten-
sion as described in the experimental procedures.

Assay for nuclease and glycosylase activity

5          $[^{32}pmdCpdG]n$ substrate which included a labeled
^{32}P 5' to mdC was prepared as follows. About 100 ng of
poly dCpdG DNA were boiled and partially annealed at
room temperature. $[\alpha^{32}P]$dCTP and cold dGTP were used for
complementary strand extension as described in the
10   experimental procedures. The free nucleotides were
separated using NAP-5 column chromatography. The puri-
fied $[^{32}pmdCpdG]n$ DNA was subjected to methylation by
SssI methylase using 320 μM AdoMet. The DNA was repuri-
fied twice using a NAP-5 column. The methylated DNA (1
15   ng) was incubated with either 30 μl DNA dMTase, nuclear
extract or buffer L. To determine whether $\alpha^{32}P$ labeled
residue is excised from the DNA it was directly applied
(3μl) onto a TLC plate. To determine whether the DNA
was demethylated it was subjected to digestion with
20   snake venom phosphodiesterase (0.2 mg in a 10μl reac-
tion volume) (Boehringer Mannheim) which attacks the
3'-OH group releasing 5'-mononucleotides. The result-
ing mononucleotides were separated on TLC plates and
autoradiographed.

25   To test whether dCTP copurifies with DNA dMTase,
which may be involved in activities other than *bona
fide* demethylation, 20 μM of dCTP with 1 μl of $\alpha^{32}P$
labeled dCTP (3000 Ci/mmole) was loaded onto the column
with nuclear extract. The ^{32}P counts were measured in
30   the flow through, washes and in the different frac-
tions. About 1.1 million counts were loaded onto the
DEAE-Sepharose column and were all recovered up to
fraction 8.

          To determine whether DNA dMTase contains a DNA
35   polymerase activity, DNA demethylase reactions were

performed in presence of 500 μM of ddCTP (Pharmacia) or
500 μM of m5dCTP (Boehringer Mannheim) at initial rate
conditions.

5   To determine whether DNA dMTase is sensitive to
RNase or Proteinase K treatment, DNA dMTase was pre-
treated for 1 h at 56°C with 200 μg/ml proteinase K
(Sigma). A demethylation reaction was carried out with
this pretreated fraction in the usual manner using both
demethylation assays described in the experimental pro-
10   cedures.   To test the effect of RNA digestion on the
demethylation reaction, the fractions from different
columns were treated with 100 μg/ml RNase A (Sigma).

Demethylation of pBluescript SK(+) Plasmid

       About 4 μg plasmid pBluescript SK (Stratagene)
15   was subjected to methylation using SssI methylase. The
methylated plasmid (4 ng) was incubated with 30 μl of
DNA dMTase Fraction 4 of DEAE-Sephacel column under
standard conditions, extracted with phenol: chloroform
and precipitated with ethanol. About 1 ng of the plas-
20   mid were subjected to digestion with 10 units each of
either of the restriction endonucleases EcoRII (GIBCO-
BRL), DpnI, HhaI or HpaII (New England Biolabs) before
and after methylation as well as after DNA dMTase
treatment in a reaction volume of 10 μl for 2 hour at
25   37°C.   Following restriction digestion the plasmids
were extracted with phenol:chloroform, ethanol precipi-
tated and resuspended in 10 μl.   The plasmids were
electrophoresed on a 0.8% (w/w) Agarose gel, trans-
ferred onto a Hybond Nylon membrane and hybridized with
30   pBluescript SK(+) plasmid which was ^{32}P labeled by ran-
dom-priming (Boehringer Mannheim).

**Effect of Redox Reagents (NAD, NADH, NADP, NADPH and
FeCl$_3$) on demethylase activity**

       The reagents were prepared at 100 μM concentra-
35   tion and added at a final concentration of 10 μM to a
standard methyl removal assay under initial rate condi-

tions as described in the experimental procedures. The
methyl removal activity in presence of each of the
cofactors was compared to a control DNA dMTase reac-
tion.

5   **Determination of kinetic parameters**

        For determination of kinetic parameters, the
demethylation reactions were performed using both
assays (generation of dCMP and removal of methyl) as
described in the experimental procedures except that
10 varying DNA concentrations from 0.1 nM to 2.5 nM were
used in a total volume of 50μl including 30 μl of DNA
dMTase.   Since it has been established by previous
experiments that the reaction proceeds for at least 3
hours, the initial velocity of reaction was measured
15 at one hour intervals. The velocity data was collected
at each substrate DNA concentration range stated for
both assays.   The Km and Vmax values for DNA demethy-
lase activity were determined from double reciprocal
plots of velocity versus substrate concentration.

20 **Measurements of methanol production catalyzed by
    demethylase by gas chromatography**

        Gas chromatography was performed with a Varian™
model 3400 GC equipped with a 30m Stabilwax™ column
(0.053 cm i.d.: Restek Corporation).   Nitrogen™ was
25 used as carrier gas at a flow rate of 32 ml/min, the
injector and detector chambers were at 200 and 300°C
respectively. The column was maintained at 40°C for 5
minutes after sample injection.

        The demethylase reaction was performed in eppen-
30 dorf tubes kept within sealed scintillation vials with
300 μl of water as aqueous phase (in radioactive trap-
ping experiments this was replaced by 300 μl of metha-
nol).   The demethylase reaction was initiated in buffer
L (10 mM MgCl$_2$, 10 mM Tris-HCl pH 8.0) with 500 ng of
35 tritiated SK plasmid (6000 dpm/μl) and 100 μl of
demethylase at 37°C. After overnight incubation at 37°C,

the aqueous phase surrounding the eppendorf tube was
transferred to a fresh eppendorf tube, 2 µl of this
mixture was injected in the gas chromatography using a
gas tight syringe (Hamilton, Reno, Nevada).

5   **Coupled *in vitro* transcription translation**

The mRNAs encoded by the pcDNA 3.1/His Xpress
demethylase constructs described above were transcribed
and translated by coupled transcription-translation
using Promega™ TNT reticulocyte lysate kit (according
10 to manufacturer's protocol), 2 µg of each construct and
40µCi of [^{35}-S]methionine (1,000Ci/mmol, Amersham) in a
50µl reaction volume. To purify non labeled *in vitro*
translated demethylase, coupled *in vitro* transcription
and translation was performed as above but in the pres-
15 ence of cold methionine. The translation products were
bound to a Probond™ nickel column (Invitrogen) and
demethylase was eluted according to the manufacturer's
protocol with increasing concentrations of imidazole.
Demethylase is eluted at 350-500mM imidazole. The imi-
20 dazole eluted demethylase was dialyzed and concentrated
by lyophilization.

**Gas chromatography coupled with Mass spectrometry (GC-
MS) Analyses for identification of volatile product of
25 demethylase catalyzed reaction as methanol**

The demethylation reactions (volume 50 1) were
run in conical vials having a total internal volume of
350 microlitres. The vials were closed with a teflon-
lined screw cap and left at room temperature for 18 h.
30 The vials were cooled in an ice bath, opened and 10 mg
of NaCl and 50 microlitres of toluene were added. The
vials were frequently shaken over a period of 1 h. The
toluene phases were pipetted into clean vials in a man-
ner to rigorously exclude water carry over. Anhydrous
35 sodium sulfate (5 mg) was added to the toluene extracts
to remove water, and the toluene phases were pipetted

into autoinjector vials for GC/MS analysis.   Aliquots
of 3 microlitres were analyzed under the following
instrumental conditions: Instrument: Hewlett-Packard
5988A; Column: 30 m x 0.25 mm i.d. fused quartz capil-
5   lary with 0.25 micron DB-1 liquid phase, programmed
after an initial hold for 1 min at 70 deg at 5 deg/min
to 80 deg, then ramped ballistically to 280 deg for
bake-out for 5 min;   Injector and interface tempera-
tures: 250 deg;   Helium flow rate 1.5 ml/min; Mass
10   spectrometer: ion source 200 deg, 70 eV electron impact
ionization, scanning from m/z 10 to 50 in full scan
mode was begun 6 s after injection, and ceased at 1.5
min to avoid acquisition of the intense toluene solvent
peak.
15
**Human A549 cells bear a demethylase activity that could
be purified away from dCTP and DNA MeTase**
        The use of an appropriate cellular source and a
direct assay for demethylase activity are obviously
20   critical. As we have previously shown that demethylase
activity was induced in response to ectopic expression
of the Ras oncogene (Szyf et al., 1995) we reasoned
that cancer cells might bear high levels of demethy-
lase activity.   Based on preliminary studies demon-
25   strating the presence of high levels of demethylase
activity in the human lung carcinoma cell line A549, we
have chosen this cell line for our further studies and
purification steps. Previous studies have used indi-
rect measures such as increased sensitivity to methyla-
30   tion-sensitive restriction enzymes as indicators of
demethylase activity (Weiss et al., 1996; Jost et al.,
1995).   To directly measure the conversion of 5-mdCMP
in DNA to dCMP, we have utilized a completely methyl-
ated ^{32}P labeled [mdC^{32}pdG]n double stranded oligomer
35   which we had previously described (Szyf et al., 1995).
Following incubation with the different fractions, the

DNA is purified and subjected to cleavage with microco-
cal nuclease to 3' mononucleotides. The 3' labeled
mdCMP and dCMP are separated by thin layer chromatogra-
phy (TLC) and the conversion of mdCMP to dCMP is
5   directly determined. This assay provides a stringent
test for bona fide demethylation and discriminates it
from previously described 5mCpC replacement activities
(Jost et al., 1995; Weiss et al., 1996). The glyco-
sylase-demethylase activity described by Jost et al.
10   (Jost et al., 1995) will require the presence of a
ligase activity and an energy source for replacement of
mdC with C to be detected by our assay, whereas the
demethylase activity described by Weiss et al. will not
be detected since it replaces the intact mdC^{32}pdG dinu-
15   cleotide with a cold dCpdG without altering its state
of methylation (Weiss et al., 1996).

Nuclear extracts were prepared from A549 cells,
applied onto a DEAE-Sephadex column, eluted with a lin-
ear gradient from 0.2-5.0M NaCl and the fractions were
20   assayed for demethylase (dMTase) activity as described
in the experimental procedures. As shown in Fig. 1(A)
a clear peak of dMTase activity is eluted at the high
salt fraction 10.

Conversion of methylated cytosine to cytosine:
25   Nuclear extracts prepared from A549 cells (1.1 mg) were
passed through an AMICON™ 100 spin column. The retain-
ant (98.56 mg, 0.2 mg/ml) was loaded onto a DEAE-Sepha-
rose column, the different chromatographic column frac-
tions eluted by a linear NaCl gradient (0.2-5M) were
30   desalted and (30 μl) incubated with 1 ng of [mdC^{32}pdG]n
double stranded oligomer for 1 hour at 37°C, digested
to 3' mononucleotides and analyzed on TLC as described
in the experimental procedures. Control methylated
(ME) and nonmethylated (NM) [dC^{32}pdG]n substrates were
35   digested to 3' mononucleotides and loaded on the TLC

plate to indicate the expected position of dCMP and
mdCMP. The active fraction is indicated by an arrow.
This fraction was loaded on S-Sepharose followed by Q-
Sepharose and DEAE-Sephacel fractionation.

5   The first chromatography step purified the
dMTase activity from the bulk of nuclear protein
(Fig. 1B) and is a very effective purification step.
           DNA dMTase activity as measured by the release
of volatile methyl residues. The different column
10   fractions were incubated with 1ng (4 x 10^6 dpm/μg) of
[^{3}H]-CH$_3$-[mdCpdG]n oligomer and the release of volatile
methyl residues was determined (-) and presented as
total dpn). The results are an average of three inde-
pendent determinations. Protein concentration was
15   determined using the Bio-Rad Bradford kit (-). The
elution profile of 20 μM of [^{32}P]-α-dCTP incubated with
the protein was determined by scintillation counting of
the different DEAE fractions (-) and presented as frac-
tion of dCTP loaded on the column.

20   To exclude the possibility that the DNA dMTase
activity detected in our assay is carried by the DNA
MeTase, we assayed the fractions for DNA MeTase activ-
ity using a hemimethylated DNA substrate as previously
described (Szyf et al., 1991). As observed in Figure
25   1B DNA MeTase activity is detected in the second and
third fractions, thus our fractionation separated DNA
dMTase away from the DNA MeTase suggesting that they
are independent proteins.

           There is a remote possibility that the demeth-
30   ylation observed is not a bona fide demethylation but
a consequence of a glycosylase removal of mC, followed
by removal of the remaining deoxyribose-phosphate by AP
(apyrimidine) nuclease, repair of the gap catalyzed by
DNA polymerase using trace dCTP contained in the frac-
35   tion and ligation of the break with ligase in the pres-

ence of residual ATP. For this hypothesis to be con-
sistent with our data, four independent enzymes and two
cofactors have to cofractionate with DNA dMTase.   To
exclude the possibility that a trace amount of dCTP is
5   bound to DNA dMTase active fraction, we have added 20
μM of ^{32}P labeled dCTP (10×10^6 cpm) to the nuclear
extract and determined its elution profile on the DEAE
column.   Less than background cpm (10 cpm) were
detected in the DNA dMTase active fraction suggesting
10   that our first column purifies dCTP away from the DNA
dMTase at least 1×10^6 fold (Fig. 1B). If any dCTP is
present in the nuclear extract, the remaining concen-
tration after fractionation on DEAE is well below the
Kms of the known DNA polymerases. The possibility that
15   dCTP is so tightly bound to the enzyme that it could
not be replaced by the exogenous ^{32}P labeled dCTP is
very remote since an enzyme using dCTP as substrate
must readily exchange dCTP.
      The active fraction 10 was further fractionated
20   sequentially on the following columns: S-Sepharose and
Q-Sepharose.   The DNA dMTase eluted at the high salt
fraction from both columns as determined by the
[mdC^{32}pdG]n demethylation assay (Fig. 1A).   The ion
exchange chromatography was followed by chromatography
25   on DEAE-Sephacel.
      The fact that we have maintained our activity
even after 4 fractionation steps (Table 1) and that
only a single polypeptide is apparent after the last
purification step argues strongly against the possibil-
30   ity that the activity detected in our study is a repair
or replacement activity.   Any replacement mechanism
must involve a number of proteins and additional cofac-
tors and substrates. In summary, the chromatography of
the demethylase activity in A459 cells provides strong

support to the hypothesis that mammalian cells bear a
bona fide demethylase activity.

DNA dMTase releases a volatile derivative

A *bona fide* demethylation has to result in
5   release of the methyl group as a volatile derivative
such as CO_2, methanol, methane or formaldehyde.   We
have therefore incubated a $\{[^3H]$ -CH_3-dCpdG$\}$n   double
stranded oligonucleotide with the different column
fractions and the rate of release of the tritiated
10 methyl from the aqueous phase was determined by scin-
tillation counting of the remaining radioactivity in
the reaction mix.   As demonstrated in Fig. 1b (dia-
mond), the dMTase active fractions release labeled
methyl groups from the methylated substrate.

15 **DNA dMTase is a protein which is inhibited by RNA, does
not involve an exchange activity and does not require
additional cofactors**

DNA dMTase activity measured either as transfor-
20 mation of mdC to C (Fig. 2a) or as release of volatile
methyl residues (Fig. 2c) is abolished after proteinase
K treatment and is not inhibited but rather enhanced
following RNase treatment.   500 μM of ddCTP which
inhibits DNA polymerase   does not inhibit   demeth-
25 ylation of the [mdC32pdG]n substrate, nor is it inhib-
ited by high concentrations of   methyl-dCTP (500 μM)
(Fig. 2a), which is consistent with the hypothesis that
demethylation does not involve an excision and replace-
ment mechanism.   If a replacement mechanism is involved
30 in demethylation, the presence of mdCTP should result
in incorporation of methylated cytosines and essential
inhibition of demethylation.   Thus, the DNA dMTase
identified here is a protein and not an RNA and is une-
quivocally different from the previously published RNA
35 based or glycosylase based demethylase activities.

        The DNA dMTase reaction proceeds without any
requirement for additional substrates such as dCTP,
redox factors such as NADH and NADPH or energy sources
such as ATP (data not shown). As observed in Fig. 2b
5 and 2d, the DNA dMTase reaction maintains its initial
velocity up to 90 minutes and continues up to 120 min-
utes. This time course is inconsistent with dependence
on enzyme-bound additional nonreplenishable substrates
such as dCTP or ATP or a nonreplenishable redox factor
10 such as NADH or NADPH. Exhausting the nonreplenish-
able substrate or redox factor would have resulted in
rapid deceleration of the initial velocity.

A product of the demethylation reaction is deoxyCyto-
15 **sine in DNA**

        What is the product of the demethylation reac-
tion? The results presented above (Fig.1a, 2a and b)
based on a one dimension TLC separation show that DNA
dMTase generates dC from mdC in DNA. To further sub-
20 stantiate this conclusion, we subjected DNA dMTase
treated DNA to remethylation with the CpG MeTase M.*Sss*
I which can transfer a methyl group exclusively to dC.
The results presented in Fig. 3a show that the demeth-
ylated product of DNA dMTase is dC since it is com-
25 pletely remethylated with M.*Sss* I. The identity of the
demethylated product as dC was further established by a
two-dimension TLC analysis demonstrating that the prod-
uct of dMTase comigrates with a cold dCMP standard in
both dimensions (Fig. 3b).
30   DNA dMTase does not release a nucleotide, a
phosphorylated base or phosphate from methylated DNA
when incubated with a [32pmdCpdG]n substrate which
included a labeled 32P 5' to mdC or our standard meth-
ylated substrate (Fig.1) where 32P is 3' to the m5dC
35 (Fig. 3c). Nuclear extracts which obviously contain a
number of glycosylases and nucleases release phospho-

rylated derivatives in the same assay (Fig. 3c).
dMTase transforms the methyl cytosine in the
[32pmdCpdG]n substrate to cytosine as demonstrated
when the reacted DNA is digested to 5' mononucleotides
5   (Fig. 3c +V PDS) and analyzed by TLC.   Since this
reaction does not involve release of a 32P derivative
(Fig. 3c -V PDS), it demonstrates that dMTase trans-
forms methylated cytosines to cytosines on DNA without
disrupting the integrity of the DNA substrate by glyco-
10 sylase or nuclease activity .

The second product of the dMTase reaction is methanol
        What is the identity of the leaving group?   The
results presented in Fig1b suggest that the labeled
15 methyl leaves the DNA as a volatile compound.   The
demethylase reaction involves release of the methyl
group *per se* whereas the cytosine base ring remains in
the aqueous phase.   Fig. 4a demonstrates this point by
using a methylated plasmid labeled with a ^{3}H-hydrogen
20 at the sixth position of cytosine and [14C]-methyl at
the fifth position of cytosine as a substrate.
        The three most obvious candidates the methyl
group is leaving as are formaldehyde, carbon dioxide,
and methanol.   Methadone trapping for labeled formalde-
25 hyde detection and sodium hydroxide trapping for
labeled carbon dioxide detection were both negative in
identifying the form in which the methyl group is leav-
ing in the dMTase reaction (data not shown).   The other
possible chemical form that the methyl group may leave
30 the DNA as, is methanol.   Since methanol is a volatile
compound, a simple method to measure generation of
methanol is a scintillation-volatilization assay (see
Fig. 4b for description).   Volatilization assays have
been previously used to measure release of methanol in
35 demethylation reactions.   The demethylation reaction
mix containing the labeled $\{[^3H]$ -CH$_3$-dCpdG$\}$n   substrate

with either dMTase or no enzyme, as a control, is added
to an uncapped 0.5 ml tube which is placed in a sealed
scintillation vial containing scintillation fluid.
Released methanol is volatile, diffuses out of the open

5   reaction tube and is mixed with the excess of the scin-
tillation fluid in the vial registering as counts in
the scintillation counter. As a control indicating
that methanol is volatilized under the conditions of
our assay, we incubated approximately equal counts of

10   radioactively labeled methanol under the same condi-
tions and measured the counts in a scintillation coun-
ter at different time points. As observed in Fig. 4c
the majority of methanol in the reaction tube volatil-
izes from the reaction tube into the scintillation

15   fluid following an overnight incubation at 37°C. The
experiment shown in Fig. 4b demonstrates that volatil-
ized label is released from methylated DNA only in the
presence of dMTase.

        The identity of the volatile group has been

20   determined to be methanol by a gas chromatography (GC)
analysis. The demethylation and control reactions
(indicated in Fig. 4e) were performed in an uncapped
tube placed in a sealed scintillation vial containing a
larger volume (300μl) of water. The volatile residue

25   diffuses into the surrounding water and mixes with it.
A 2 μl sample of the surrounding water was injected
into a GC column as described in the methods. As
shown in Fig. 4e, the volatile compound released by
dMTase in a dose response manner coelutes with metha-

30   nol. Release of methanol is observed only in the pres-
ence of both dMTase and methylated DNA. No methanol is
released when dMTase is reacted with nonmethylated DNA,
demonstrating that methanol is a product of demethyla-
tion of DNA.

        The leaving group was also identified as metha-
nol using gas chromatography coupled with Mass spec-
trometry (GC-MS). As illustrated in Fig. 4f., incuba-
tion of methylated DNA with dMTase (dMTase+ME-DNA)
5   results in release of a peak with the retention time
and mass spectrum (peaks are identified at 32 and 29
atomic mass which are the atomic masses of methanol and
ionized methanol respectively) which is consistent with
its identification as methanol. Incubation of dMTase
10   with nonmethylated DNA does not release methanol indi-
cating that methanol is a product of the demethylation
reaction. No methanol is released when the samples are
incubated with dMTase treated with protease K indicat-
ing that the release of methanol from methylated DNA is
15   catalyzed by an enzymatic activity.

Demethylation involves transfer of a hydrogen from
water to regenerate cytosine
        If demethylation involves removal of the methyl
20   moiety from mdC, a hydrogen has to be transferred to
the carbon at the 5' position to regenerate cytosine.
Since no redox factors are involved, what is the source
of the hydrogen? To test the hypothesis that the
source of the hydrogen is water, we incubated either
25   non labeled [mdCpdG]n or [dCpdG]n double stranded DNA
with DNA dMTase for different time periods in the
presence of tritiated water, following which the DNAs
were digested to 3' dNMPs, separated on TLC with non-
radioactive standards for each of the 5 possible dNMPs
30   and exposed to a tritium sensitive phosphorimaging
plate. As seen in Fig.4d, dMTase catalyzes the trans-
fer of a tritiated hydrogen from water to dCMP in meth-
ylated DNA in a time dependent manner only when meth-
ylated DNA is used as a substrate. Based on the
35   experiments described in Fig.3 and 4 we propose that
dMTase catalyzes the exchange of the methyl group at

the 5' position of cytosine in DNA with hydrogen from
water and the methyl group reacts with the remaining
hydroxyl group to form methanol (Fig. 5).

5   **Substrate and sequence specificity of DNA dMTase**
        Methylation of CpG dinucleotides is the most
characterized modification occurring in genomic
DNA8,48. The results presented in Fig.6 demonstrate
that DNA dMTase is a general DNA dMTase activity that
10 demethylates fully or hemimethylated dCpdG in DNA
flanked by a variety of sequences which are distributed
at different frequencies, but does not demethylate
methylated adenines or methylated cytosines that do not
reside in the dinucleotide CG.   First, as shown in
15 Fig.6a, a plasmid DNA methylated *in vitro* at all dCpdG
sites with M.*Sss* I and all d*CdCdGdG sites with M. *Msp*
I   (which methylates the external C in the sequence
*CCGG, thus enabling the determination of demethylation
at the CC dinucleotide) and in *vivo* with the *E. coli*
20 DCM MeTase at dCmdCdA/dTdGdG sites and with the DAM
MeTase at dGmdAdTdC sites (adenine methylated) was
treated with dMTase and the state of methylation of the
plasmid was determined using the indicated methylation
sensitive restriction enzymes.   dMTase demethylates C*G
25 methylated sites as indicated by the sensitivity of the
dMTase treated plasmid to *Hpa* II and *Hha* I but does not
demethylate C*C,C*A or C*T methylated sites as indi-
cated by the resistance to *Msp* I and *Eco* RII restric-
tion enzymes, or adenine methylation as indicated by
30 its sensitivity to *Dpn* I. Second, bisulfite mapping
analysis of methylation of 5 methylated C*G sites
residing in a M.*Sss* I *in vitro* methylated pMetCAT plas-
mid following dMTase treatment shows that all C*G sites
are demethylated irrespective of their flanking
35 sequences thus excluding the possibility that demeth-
ylation is limited to CCGG or CGCG sequences (Fig. 6b).

Third, dMTase does not demethylate two fully methylated
cytosine bearing oligomers [dmC32pdA]n, [mdC32pdT]n
demonstrating that mdCpdA and mdCpdT are not demethyl-
ated by DNA dMTase (Fig. 6d). Fourth, dMTase demethyl-
5   ates a hemimethylated synthetic substrate
[dCpdG]n*[mdC32pdG]n (Fig. 6d). Demethylation of SK is
complete under these conditions (Fig. 6a) whereas
demethylation of a methylated [mdCpdG]n substrate is
not complete under the same conditions (Fig. 6d). This
10 can reflect differences in the sequence composition of
the substrate and the frequency of methylated cyto-
sines. The [mdCpdG]n contains on average 16 fold more
methylated cytosines per molecule than plasmid DNA.
Alternatively, these differences might reflect discrep-
15 ancies in the assays used, restriction enzyme digestion
versus a nearest neighbor analysis. To address this
discrepancy we have labeled a fully methylated SK plas-
mid with [α^{32}P]dCTP, 5-methyl-dCTP and the other dNTPs,
subjected it to dMTase treatment and digested it to
20 mononucleotides at different time points following the
initiation of the reaction and subjected the samples to
a TLC analysis. As shown in Fig. 6c, the SK plasmid is
fully demethylated at 3 hours which is consistent with
the results obtained with methylation sensitive
25 restriction enzymes (Fig. 6a).
       The Km of DNA dMTase for hemimethylated and
fully methylated DNA was determined by measuring the
initial velocity of the reaction at different concen-
trations of substrate (Table 2). The calculated Km for
30 hemimethylated DNA is 6 nM which is two fold higher
than the Km for DNA methylated on both strands, 2.5-3
nM (Table 2). It is unclear yet whether this small
difference in affinity to the substrate has any sig-
nificance in a cellular context. Thus similar to the
35 DNA MeTase DNA dMTase shows dinucleotide sequence

selectivity but in difference from DNA MeTase which shows preference to hemimethylated substrates dMTase prefers fully methylated DNA which is consistent with a role for DNA dMTase in altering established methylation patterns.

Table 1

Purification of DNA dMTase

Purification step	Total protein (µg)	Total dpm	pMole/µg	pMole/µg/h	Fold Purification
Nuclear extract	6000	1107.2	5.5×10^{-5}	1.833×10^{-5}	-
DEAE-Sephadex	3.75	5844	0.4674	0.156	8445.5
SP-Sepharose	0.77	5106	1.989	0.663	35939.84
Q-Sepharose	0.46	5335	3.4	1.13	62860.65
DEAE-Sephacel	0.018	1834	30.57	10.19	552243.2

Table 2

Kinetic parameters for DNA dMTase

Method	K_m (DNA)	V_{max} (pMole/h)
Methylated oligo CpG	2.5 nM	340
Hemi-methylated CpG	6.0 nM	402
Methylated SK-DNA	3.3 nM	40.42

Cloning and construction of demethylase expression vectors

PCR amplification of the MBD domain of the putative demethylase candidate cDNA

One µg of total RNA prepared from the human small lung carcinoma cell line A549 was reverse tran- scribed using Superscript reverse transcriptase and random primers (Boehringer) in a 25 µl reaction volume according to conditions recommended by the manufacturer (GIBCO-BRL). Five µl of reverse transcribed cDNA were subjected to an amplification reaction with Taq poly- merase (Promega, 1 unit) using the following set of

primers: sense 5'CTGGCAAGAGCGATGTC 3' SEQ ID NO:9, antisense 5'AGTCTGGTTTACCCTTATTTTG 3' SEQ ID NO:10.

Amplification conditions were: step 1. 95°C 1 min.; step 2: 94°C 0.5 min; step 3: 45°C 0.5 min.; step 4: 72°C 1.5 min; steps 2-4 were repeated 30 times. $MgCl_2$ was adjusted to 1 mM according to conditions recommended by the manufacturer. The PCR products were cloned in pCR2.1 vector (InVitrogen) and the sequence of the cDNAs was verified by dideoxy-chain termination method using a T7 DNA sequencing kit (Pharmacia). The amplified fragment was excised from the plasmid with EcoRI, labeled with a Boehringer random prime labeling kit according to manufacturer's protocol and alpha ^{32}P-dCTP. The labeled probe was used to screen a HeLa cell cDNA library in λTriplEx phage (Clontech) according to standard procedures. Positive clones were identified and further purified by serial dilutions for 4 rounds. The insert in the pTriplEx plasmid was excised from the phage according to manufacturer's protocols and the identity of the insert was verified by sequencing. The insert was excised by NotI restriction and subcloned into either the inducible expression vector: Retro tet on (Clontech) in the sense and antisense orientation or the pcDNA3.1/His Xpress vector in all three frames and in the antisense orientation.

Transfection and expression of demethylase in vertebrate cells

Ten μg of either Retro tet on demethylase or pcDNA 3.1/His Xpress demethylase are mixed with 8 μl of transfection lypophilic reagent Pfx-2 (Invitrogen) and placed upon 100,000 mouse (3T3 Balb/c, human (A549) or monkey cells (CV-1) according to manufacturer's protocol in OPTIMEM medium for 4 hours. Cells are harvested after 48 hours and demethylation and demethylase activity is determined by measuring total genomic DNA meth-

ylation using standard techniques or a cotransfected *in vitro* methylated plasmid using a HpaII /MspI restriction enzyme analysis. Cellular transformation is measured by a soft agar assay.

5   **Demethylation of pBluescript SK(+) Plasmid**

About 4 µg plasmid pBluescript SK (Stratagene) was subjected to methylation using SssI methylase. The methylated plasmid (4 ng) was incubated for different
10   time points as indicated with 30 µl of DNA dMTase Fraction 4 of DEAE-Sephacel™ column under standard conditions, extracted with phenol: chloroform and precipitated with ethanol. About 1 ng of the plasmid were subjected to digestion with 10 units each of either of
15   the restriction endonuclease EcoRII (GIBCO-BRL), DpnI, or HpaII (New England Biolabs) before and after methylation as well as after DNA dMTase treatment in a reaction volume of 10 µl for 2 hour at 37°C. Following restriction digestion the plasmids were extracted with
20   phenol:chloroform, ethanol precipitated and resuspended in 10 µl. The plasmids were electrophoresed on a 0.8% (w/w) Agarose gel, transferred onto a Hybond™ Nylon membrane and hybridized with pBluescript SK(+) plasmid which was ^{32}P labeled by random-priming (Boehringer
25   Mannheim).

dMTase activity coelutes with a ~45 KDa polypeptide when sized under denaturing conditions but migrates as a higher molecular weight complex under non denaturing
30   **conditions.** dMTase was purified up to 500,000 fold by four chromatographic steps (Table 1). We first determined the identity of the polypeptide associated with dMTase activity by SDS-PAGE analysis of the active fractions. As observed in Fig. 7a, a cluster of 4
35   polypeptide bands from ~44 KDa to 35 KDa coelute with dMTase activity in the last two chromatographic steps

(the lower fragment might be a degradation product as evidenced by its abundance in the later chromatographic steps). However when the active DEAE-Sephacel fraction is size fractionated on a 4% non denaturing acrylamide

5   column, the dMTase activity elutes at the high molecular weight of ~170 KDa (Fig. 7c, fraction 63). SDS-PAGE analysis of this fraction (63) reveals only two bands (Fig. 7b) observed in the active chromatographic fractions (Fig. 7a)   To further determine whether

10   dMTase is found in a multimeric complex, fraction 63 was size fractionated on a glycerol gradient (Fig. 7d) and DNA dMTase activity eluted at the ~170 kDa range. As only two main small polypeptides were identified in fraction 63 (approximately 35-43 KDa), dMTase is proba-

15   bly found in either a homomeric complex if only one of the two peptides is dMTase or a heteromeric complex if both polypeptides are associated with dMTase activity.

a.   Identification of a lead DNA dMTase candidate by
20   **homology search of dbEST**

As the purification of dMTase suggests that the dMTase is of very low abundance, only ~19 ng of dMTase could be isolated from 6 mg of nuclear extract (Table 1), we opted for cloning the dMTase based on its

25   following functional properties. First, since dMTase specifically demethylates methylated CG dinucleotides, we assumed that it should bear the ability to recognize methylated CG dinucleotides. Second, the demethylase transforms methylated cytosine in DNA to cytosine.

30   Third, the demethylase releases the methyl group as a volatile compound.

Previous reports have shown that proteins interacting with methylated DNA share a common domain (MDBD). A TBLASTN search of the dbEST database identi-

35   fied a novel expression tag cDNA (from a T-cell lymphoma Homo sapiens cDNA 5' end) (gb/AA361957/AA361957

EST71295) and the mouse homologue ((gb/W97165/W97165
mf90g05.r1) from Soares mouse embryo NbME13.5) with
unknown function that bears homology to the MDBD
(Fig. 8a). A search of the GenBank database verified
5   that it is a novel cDNA that has not been included in
GenBank. Alignment of the novel EST and MeCP2 and
MeCP1 associated protein has revealed no homology
beyond the previously characterized MDBD which is con-
sistent with a different function for this methylated
10 DNA binding protein. A 201bp fragment bearing the
sequence identified in the search was reverse tran-
scribed and amplified from human lung cancer cell line
A549 RNA and was used to screen a cDNA library from
Hela cells. The largest insert cloned was of 1.36 kb
15 size and its sequence identity with the EST sequence
was determined. The cDNA is novel and has no homologue
in GenBank and no function has ever been assigned to
it. A virtual translation of the protein identified an
open reading frame (ORF) of 262 amino acids (Fig. 8b).
20 The ORF may extend further 5' as no in frame stop codon
was found upstream of this ATG. However, RACE analy-
ses and further searches of the dbEST have failed to
identify 5' sequences upstream to the one identified in
our screening.

25   A BLAST search of the candidate protein using
the Predict protein server against a database of pro-
tein domain families has identified only the MDBD
domain and found no homologue to the sequence in the
data base search. No other functional motifs were
30 identified by the Prosite analysis. This is consistent
with a novel biochemical function for this protein. A
coiled coil prediction of the sequence identified a
coiled coil domain which is known to play a role in
protein protein interactions.

The identified cDNA encodes an mRNA that is
widely expressed in human cells as revealed by a North-
ern blot analysis of human poly A+ mRNA (Fig. 8c) as
one major transcript of ~ 1.6 kb which is close to the
5   size of the cloned cDNA, verifying that the cloned cDNA
does not represent a highly repetitive RNA but rather a
mRNA encoded by a single or low copy number gene.

In vitro translated candidate cDNA bears dMTase activ-
10 **ity**

A conclusive proof for the existence of a single
protein that *bona fide* demethylates DNA is to demon-
strate that an *in vitro* translated candidate cDNA can
volatilize methyl groups from methylated DNA and trans-
15 form a methyl cytosine to cytosine in an isolated sys-
tem. The candidate dMTase cDNA was subcloned it into a
pcDNA3.1/His Xpress (INVITROGEN) expression vector in
the putative translation frame (pcDNA3.1His A) and in a
single base frame shift (pcDNA3.1His B), and was *in*
20 *vitro* transcribed and translated in the presence of
^{35}S-methionine and the resulting translation products
were resolved by SDS-PAGE. Autoradiography revealed a
~40KDa protein (Fig. 10a). The apparent size of the *in*
vitro translated protein is shorter by ~3-5 KDa from
25 the apparent size of the purified protein. The cloned
cDNA might be missing some upstream amino acids as dis-
cussed above or might be differently modified in human
cells.

Two tests established whether the *in vitro*
30 translated candidate cDNA is a *bona fide* dMTase. We
first tested whether *in vitro* translated protein
(purified on a Ni2+ charged agarose resin) can volatil-
ize and release methyl residues in [^{3}H]-CH$_{3}$-DNA using a
radioactive trapping volatilization assay. To verify
35 that the volatilized counts are true ^{3}H counts, a spec-
trum analysis was performed. As demonstrated in Fig.

10b no volatilization of tritiated methyl residues is observed in the misframe dMTase (misframe) whereas *in vitro* translated putative dMTase cDNA catalyzes the volatilization of ^{3}H-CH$_3$ residues which are trapped in

5   the scintillation cocktail.

Second, *in vitro* translated dMTase cDNA transforms CH$_3$-cytosine residing in [^{32}P]-α-dGTP labeled plasmid DNA or in [methyl-dC32pdG]n double stranded oligomer DNA to cytosine, whereas a frame shift *in*

10   *vitro* translated dMTase does not demethylate DNA (Fig. 10d). This demonstrates that the dMTase activity is dependent on the dMTase translation product and not a contaminating activity found in the *in vitro* translation kit that copurifies with the putative dMTase. The

15   reaction carried out by the *in vitro* translated dMTase displays: dependence on the dose of *in vitro* translated product (Fig. 10c), time dependence (Fig. 10d) and dependence on translated protein (Fig. 10b & d misframe, Fig. 10c protease K treatment). Taken together,

20   these results strongly suggest that the cDNA cloned here codes for a *bona fide* enzymatic DNA demethylase activity.

Transiently transfected dMTase cDNA demethylates DNA

25   dMTase cDNA and the pcDNA3.1HisC vector control were transiently transfected into human embryonal kidney cells to test whether the cDNA can direct expression of dMTase activity in human cells. The His-tagged proteins were bound to Ni2+ agarose resin and eluted

30   from the resin with increasing concentrations of imidazole. The expression of the transfected dMTase was verified by a Western blot analysis (Fig. 11b). The imidazole fractions were assayed for their ability to volatilize and release methyl residues in [^{3}H]-CH$_3$-DNA

35   using a radioactive trapping volatilization assay 1. As observed in Fig. 11a, imidazole fractions from

dMTase transfected cells volatilize[³H]-CH₃ whereas no
tritiated counts are detected in DNA treated with imi-
dazole fractions from cells transfected with a misframe
mutation of dMTase or non transfected cells. The tran-
5   siently expressed dMTase transforms methylated cytosine
in DNA to cytosine residing in two different substrates
(Figs. 11c & 11d), in a protein dependent manner (Figs.
11c & 11e), and the reaction displays substrate depend-
ence   and   saturability   (Fig.   11f).   Transiently
10 expressed dMTase was loaded on a non denaturing glyc-
erol gradient to determine its native MW.   Similar to
dMTase purified from human cells, cloned and purified
dMTase activity fractionated at the 160-190 KDa range
(data not shown).   This is consistent with self asso-
15 ciation of cloned dMTase possibly mediated by the
coiled-coil domain.

**Cloned DNA dMTase catalyzes a hydrolysis of 5-methyl-
cytosine to release methanol**

20   We determined the mechanism by which methyl
residues are released by the cloned dMTase (from Fig.
11) and compared it to the purified *bona fide* dMTase
activity.   Increasing amounts of non labeled [methyl-
dCpdG] DNA were incubated with either the *bona fide*
25 dMTase activity purified from A549 cells or the cloned
dMTase in the presence of [³H] water for 3 hours fol-
lowed by digestion to mononucleotides, a thin layer
chromatography and autoradiography. As Fig. 12a shows,
both reactions replace the methyl group in 5-methylcy-
30 tosine with a proton donated from water as indicated by
the presence of [³H] label in cytosine.

        The identity of the leaving methyl group in the
demethylation reaction catalyzed by the purified bona
fide dMTase activity was shown to be methanol.   In
35 order to identify the form that the methyl residue
leaves as in the demethylation reaction catalyzed by

the cloned dMTase an identical gas chromatography/mass
spectrometry analysis of the reaction products was per-
formed as in1. Only the properly translated form of
dMTase (both in vitro translated and transiently trans-
5   fected and purified) is able to produce ions character-
istic of methanol in a mass spectrometric analysis
(mass of 32 and 29, Fig. 12b). These results suggest
that the demethylation reaction catalyzed by the cloned
dMTase is hydrolysis of the 5-methyl-cytosine to cyto-
10   sine and methanol as described for the purified
dMTase1.

**DNA dMTase activity is undetectable in nontransformed
cells**

         The assays for dMTase activity described here
15   and the cloning of DNA dMTase cDNA enables a study of
its expression at different cellular states. Global
hypomethylation of DNA is a common observation in can-
cer cells. This has been a perplexing observation,
since DNA MeTase activity is elevated in cancer cells.
20   Hyperactivation of DNA MeTase has been proposed to play
a role in cancer development. This paradox raises
questions on the proposed role of the elevated levels
of DNA MeTase in cancer cells. One simple explanation
that has been previously suggested to resolve this
25   paradox is that cancer cells express induced levels of
DNA dMTase. We compared the DNA dMTase activity in
equal concentrations of DEAE-Sephadex fractionated
nuclear extracts (fractions 9-10) prepared from a num-
ber of carcinoma cell lines H446, Colo 205, Hela, and
30   A549 with a similar preparation from human skin fibro-
blast cells at initial rate conditions using
[mdC32pdG]n double stranded oligomer as a substrate.
As observed in Fig. 13a, whereas DNA dMTase activity is
readily observed in all carcinoma cell lines, it is
35   undetectable in nontransformed human cells. The
absence of dMTase activity in human primary cells

reflects the situation *in vivo* since dMTase activity is undetectable in preparations from different murine tis- sues whereas dMTase activity is present in a murine carcinoma cell line P19 that was transfected with the

5   H-Ras protooncogene, or human tumors carried as xeno- grafts in the same strain of mouse (Fig. 1a: COLO 205, A549. Hela). These conclusions were verified using the radioactive-trapping volatilization assay shown in Fig. 13c.

10   Since dMTase mRNA has been detected using a sen- sitive poly A+ Northern blot in all normal human tis- sues, we tested the hypothesis that the absence of detected dMTase activity in normal tissues reflects a quantitative difference in DNA dMTase mRNA between nor-

15   mal tissues and cancer lines. A Northern blot analysis and quantification of dMTase mRNA by a slot blot analy- sis shown in Fig. 13d using total RNA supports this hypothesis. Whereas minute levels of dMTase mRNA are detected in normal tissues, high levels of dMTase are

20   expressed in a murine carcinoma cell line Y1 that bears a 30 fold amplification of *Ha-ras*.

A second DNA demethylase dMTase2 identified in human and mouse

cDNA sequences, predicted amino acid sequences, and

25   GenBank accession numbers of both dMTase1 and dMTase2 from human and mouse are shown. We claim that the high level of identity of the two proteins (Figs 9c and e) suggests that the two proteins can perform the same function, DNA demethylation. The N-terminals of

30   dMTase1 and dMTase2 contain a Methylated DNA Binding Domain (MBD) and near their C-terminals is a coiled- coil domain, however the middle portions of the protein sequences have no homology to any know structural or catalytic motif. Importantly, their middle regions are

35   still extensively homologous suggesting that the cata-

lytic site of the demethylase activity lies in this
area on both proteins.

**Induced expression of DNA demethylase in the Antisense
orientation inhibits tumorigenesis *ex vivo***

5   To test the hypothesis that inhibition of DNA
dMTase can inhibit tumorigenesis tetracycline inducible
vectors carrying the human dMTase1 cDNA in either the
sense or antisense orientation were constructed and
transiently transfected into HEK 293 cells, treated for
10   48 hours either in the presence or absence of doxycy-
cline (a tetracycline analogue), selected for the last
24 hours with puromycin, and then plated on soft agar
and allowed to grow for seven days. After seven days
colonies were scored and the data presented clearly
15   show that doxycycline induced expression of the dMTase1
cDNA in the antisense orientation reduced colony forma-
tion (Fig. 15).

**Imidazole is a small molecule inhibitor of DNA
demethylase activity**

20   A template small molecule, imidazole, was tested
for the ability to inhibit DNA dMTase activity. In a
volatilization of radioactive methyl residues assay,
concentrations from $1\mu M$ to 10mM of imidazole were incu-
bated in a typical volatilization of radioactive methyl
25   residues as described above. The graph clearly demon-
strates a dose dependent inhibition of DNA dMTase
activity by imidazole, and validates a rationale for
testing imidazole based molecules as inhibitors of DNA
dMTase activity (Fig. 16).

30   **Identification of DNA demethylase cDNAs and protein
sequences**

Fig. 9a illustrates cDNA sequence of human dMTase1 (SEQ
ID NO:1) and its predicted amino acid sequence (SEQ ID
NO:2), including its Genbank location. **Fig. 9b** illus-
35   trates cDNA sequence of human dMTase2 (SEQ ID NO:3) and
its predicted amino acid sequence (SEQ ID NO:4), includ-

ing its GenBank location. **Fig. 9c** illustrates protein
sequence alignment of human dMTase1 and human dMTase2.
Fig. 9d illustrates cDNA sequence of mouse dMTase1 (SEQ
ID NO:5) and its predicted amino acid sequence (SEQ ID

5   NO:6), including its GenBank location. **Fig. 9e** illus-
trates cDNA sequence of mouse dMTase2 (SEQ ID NO:7) and
its predicted amino acid sequence (SEQ ID NO:8),
including its GenBank location. **Fig. 9f** illustrates
protein sequence alignment of mouse dMTase1 and mouse

10 dMTase2.

         While the invention has been described in con-
nection with specific embodiments thereof, it will be
understood that it is capable of further modifications
and this application is intended to cover any varia-

15 tions, uses, or adaptations of the invention following,
in general, the principles of the invention and
including such departures from the present disclosure
as come within known or customary practice within the
art to which the invention pertains and as may be

20 applied to the essential features hereinbefore set
forth, and as follows in the scope of the appended
claims.

ART 34 AMDT

WHAT IS CLAIMED IS:

1.   A DNA demethylase enzyme and/or homologue thereof, which comprises an amino acid sequence set forth in SEQ ID NOS:2, 4, 6 and 8 and homologue thereof, and wherein said DNA demethylase enzyme has about 40 KDa and is overexpressed in cancer cells.

2.   A cDNA encoding a human demethylase of claim 1, which comprises a sequence set forth in SEQ ID NOS:1 and 3.

3.   A cDNA homologous to the cDNA of claim 2, wherein said cDNA encoding mouse demethylase set forth in SEQ ID NOS:5 and 7.

4.   The use of the expression of demethylase cDNA of claims 2 or 3 to alter DNA methylation patterns of DNA *in vitro* in cells or *in vivo* in humans, animals and in plants.

5.   The use of claim 4, wherein said demethylase cDNA expression is under the direction of mammalian promoters.

6.   The use of claim 5, wherein said promoter is CMV.

7.   The use of claim 4, wherein said demethylase cDNA expression is under plant specific promoters to alter methylation in plants and to allow for altering states of development of plants and expression of foreign genes in plants.

8.   The use of claim 4, wherein said demethylase cDNA expression is in the antisense orientation to inhibit demethylase in cancer cells for therapeutic processes.

9.   The use of claim 9, wherein expression of
demethylase cDNA in mammalian cells is to alter their
differentiation state and to generate stem cells for
therapeutics, cells for animal cloning and to improve
expression of foreign genes.

10.   The use of the expression of demethylase cDNA of
claims 2 or 3 in bacterial or insect cells for produc-
tion of large amounts of demethylase.

11.   The use of the expression of demethylase cDNA of
claims 2 or 3 for the production of protein in verte-
brate, insect or bacterial cells.

12.   The use of claim 11 for producing antibodies
against demethylase.

13.   The use of the sequence of demethylase cDNA of
claim 2 as a template to design antisense oligonucleo-
tides and ribozymes.

14.   The use of the predicted peptide sequence of
demethylase cDNA of claim 2 to produce polyclonal or
monoclonal antibodies against demethylase.

15.   The use of expression of cDNA of claim 2 or 3 in
two hybrid systems in yeast to identify proteins inter-
acting with demethylase for diagnostic and therapeutic
purposes.

16.   The use of expression of cDNA of claim 2 or 3 in
bacterial, vertebrate or insect cells to produce large
amounts of demethylase for high throughput screening of

demethylase inhibitors for therapeutics and biotechnol-
ogy and for obtaining the x-ray crystal structure.

17.   A volatile assay for high throughput screening
of demethylase inhibitors as therapeutics and antican-
cer agents which comprises the steps of:
    a) using transcribed and translated demethylase
       cDNA of claim 2 or 3 *in vitro* to convert methyl-
       cytosine present in methylated DNA samples to
       cytosine present in DNA and volatilize methyl
       group;
    b) determining the absence or minute amount of
       volatilize methyl group as an indication of an
       active demethylase inhibitor.

18.   A volatile assay for the diagnostics of cancer
in a patient sample which comprises the steps of:
    a) determining demethylase activity in patient sam-
       ples by determining conversion of methyl-cyto-
       sine present in methylated DNA to cytosine pres-
       ent in DNA and volatilization of the methyl
       group released as methanol;
    b) determining the presence or minute amount of
       volatilized methyl group as an indication of
       cancer in said patient sample.

19.   Use of an antagonist or inhibitor of DNA demeth-
ylase of claim 1 or 2 for the manufacture of a medica-
ment for cancer treatment, for restoring an aberrant
methylation pattern in a patient DNA, or for changing a
methylation pattern in a patient DNA.

20.   Use according to claim 19, wherein said antago-
nist is a double stranded oligonucleotide that inhibits
demethylase at a Ki of 50nM.

21.   Use according to claim 20, wherein said oligonucleotide is
$$\left\{ \begin{array}{c} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{array} \right\}n \quad .$$
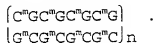
22.   Use according to claim 19, wherein the inhibitor comprises an anti-DNA demethylase antibody or an antisense oligonucleotide of DNA demethylase or a small molecule.

23.   Use according to one of claims 19 or 22, wherein the change of the methylation pattern activates a silent gene.

24.   Use according to claim 23, wherein the activation of a silent gene permits the correction of genetic defect.

25.   Use according to claim 24, wherein said genetic defect is β-thalassemia or sickle cell anemia.

26.   Use of the demethylase of claim 1, for removing methyl groups on DNA _in vitro_.

27.   Use of the demethylase of claim 1 or its cDNA of claim 2, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

28.   Use of the demethylase of claim 1 or its cDNA, of claim 2 for inhibiting methylation in cancer cells using vector mediated gene therapy.

29.   An assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase of claim 1 in a sample
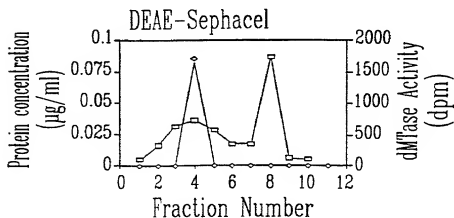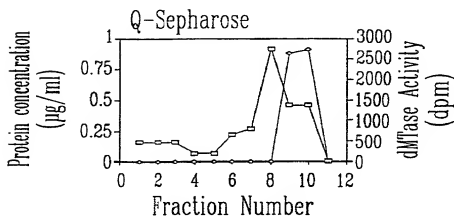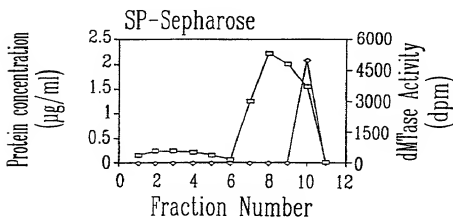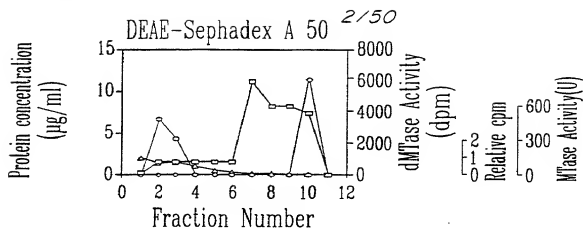
from said patient, wherein overexpression of said DNA demethylase is indicative of cancer cells.
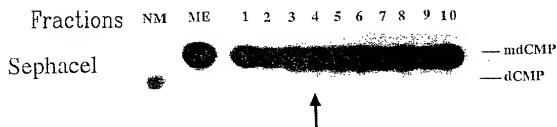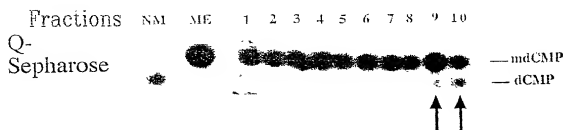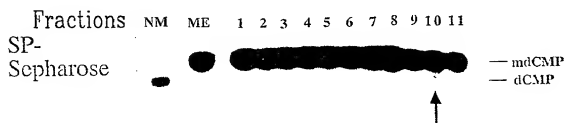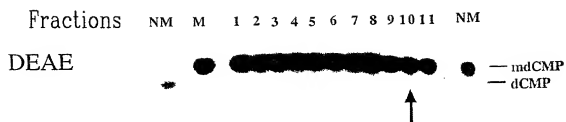
Fig. 1A

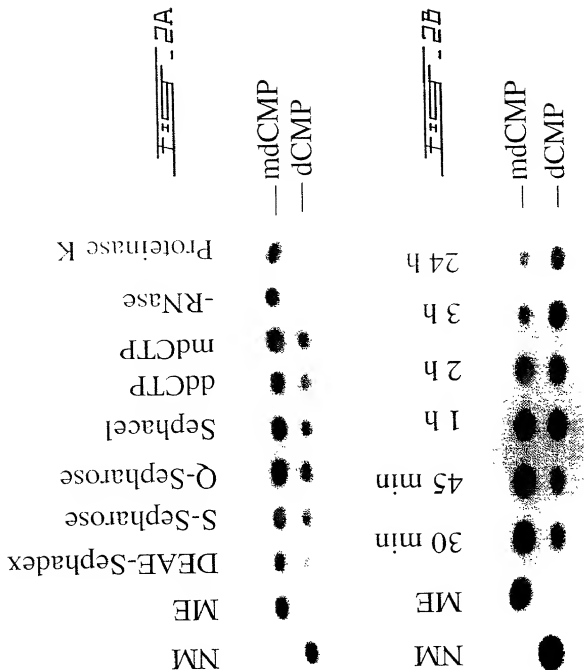DEAE-Sephadex A 50

SP-Sepharose

Q-Sepharose

DEAE-Sephacel

FIG. 1B

Fig. 2A

— mdCMP
— dCMP

Proteinase K
-RNase
mdCTP
ddCTP
Sephacel
Q-Sepharose
S-Sepharose
DEAF-Sephadex
ME
NM

Fig. 2B

— mdCMP
— dCMP

24 h
3 h
2 h
1 h
45 min
30 min
ME
NM

FIG. 2D

FIG. 2C

FIG. 3A.

FIG. 3B.

Fig. 3E

Labeled nucleotide: [α32P]-dGTP

Labeled nucleotide: [α32P]-dCTP

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

H_2O −dMTase $^{14}CH_3OH$ CH_3OH

+dMTase (20µl)   +dMTase (20µl)   +dMTase (100µl)   +dMTase (100µl)
+NM   +ME   +ME   +ME + CH_3OH

FIG. 4E

Fig. 4F

$$\triangle G = (93) - (103 + 91.5) = (-101.5) \text{ Kcal}$$
$$\text{Reaction}$$

FIG. 5

FIG. 8B

16/50

FIG. 7D

FIG. 7C

EST   DCPAIPPGWKKEEVIRKSGLSAGKSDMYYFSPSGKKFRSKPQLARYLGNTVDLS
                                                  D S
        D P LP GW ++   RKSG SAGK DVY +P GK FRSK +L Y   D
MeCP2 15 IDDPTLPEGWIRKLKQPKSGRSAGKIDVYLINEQGKAFRSKVELIAYFEKVGDIS 68

FIG. 8A
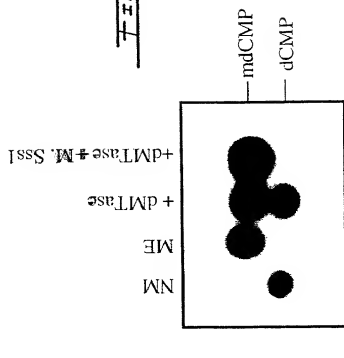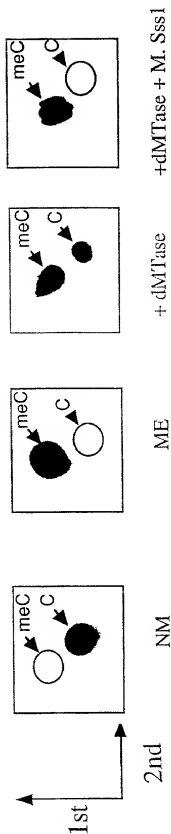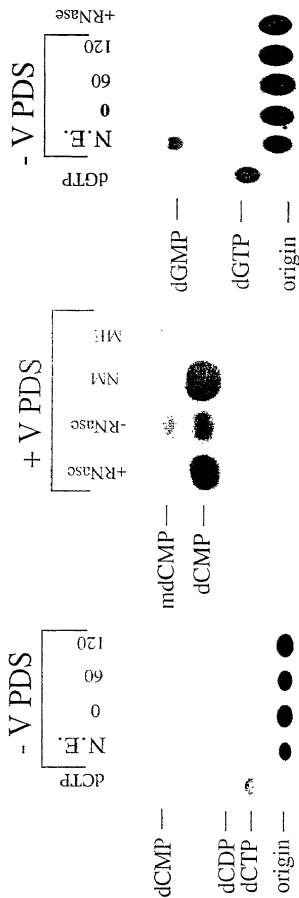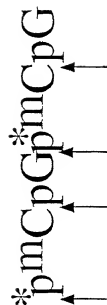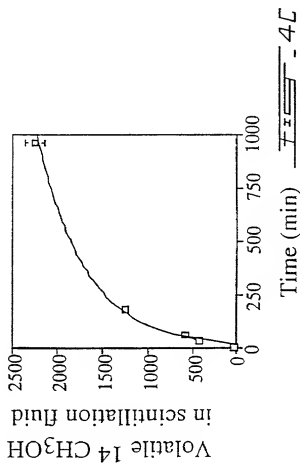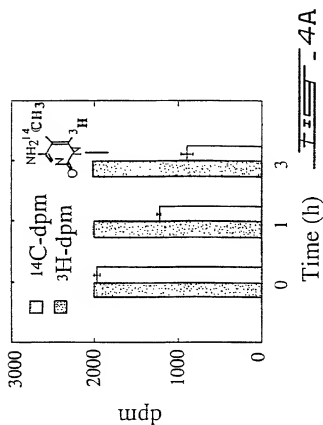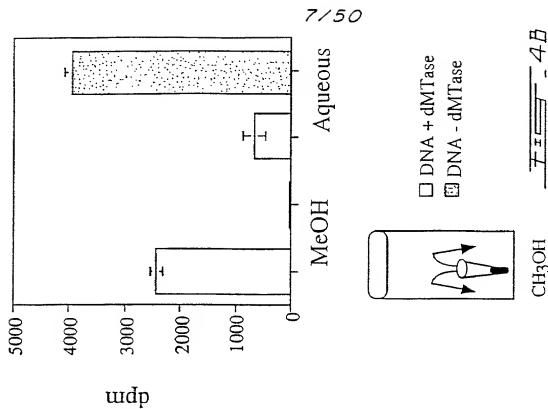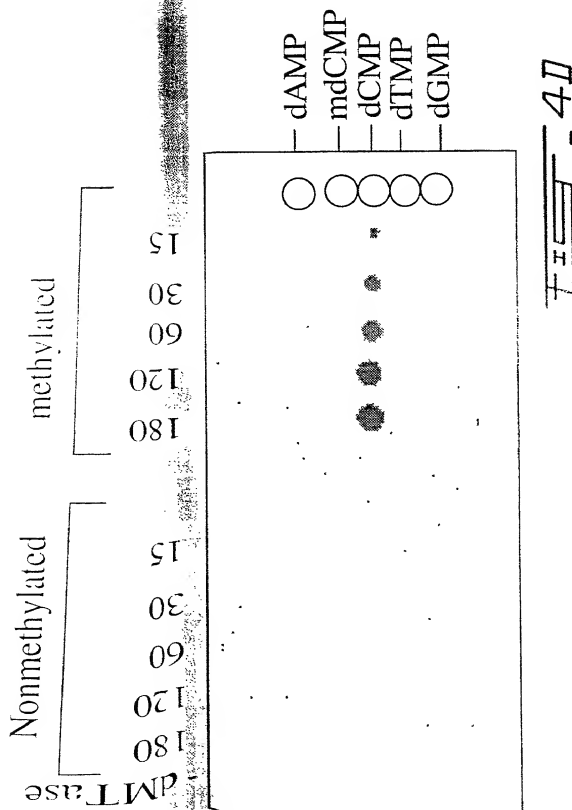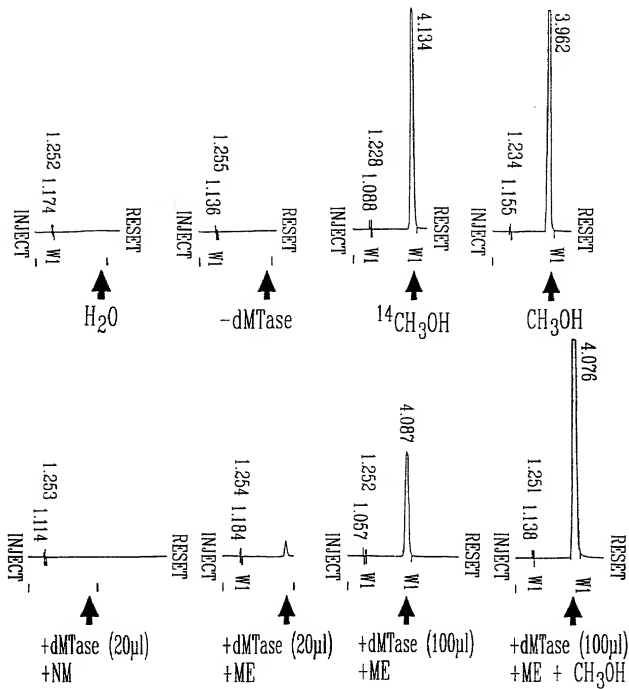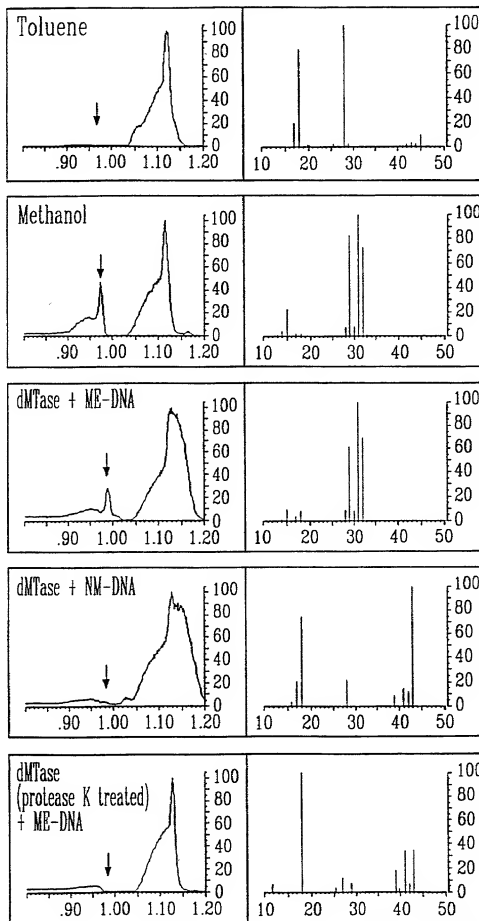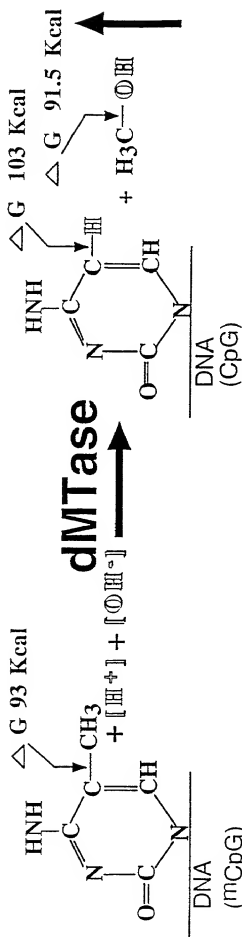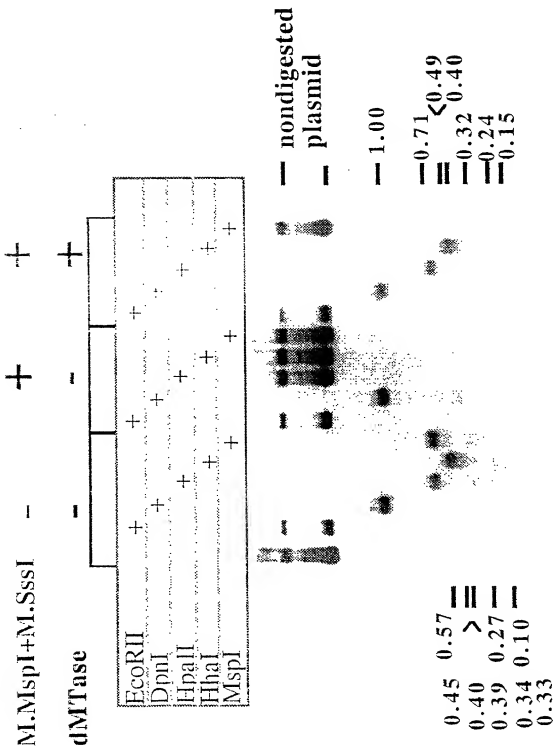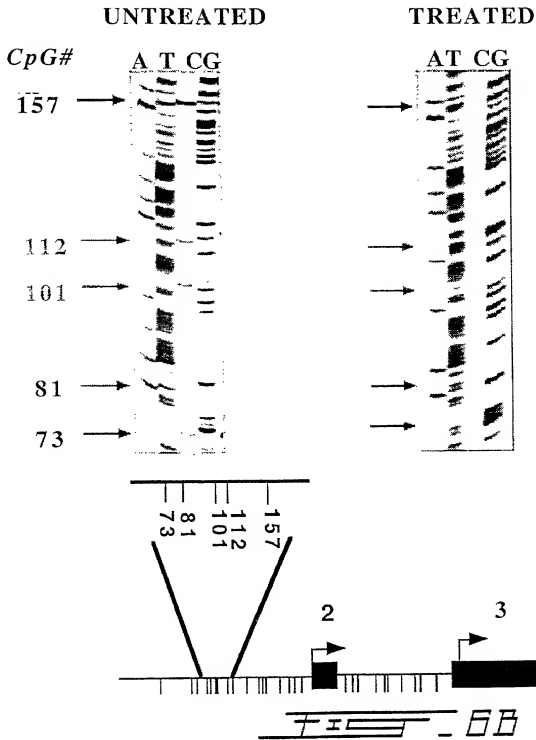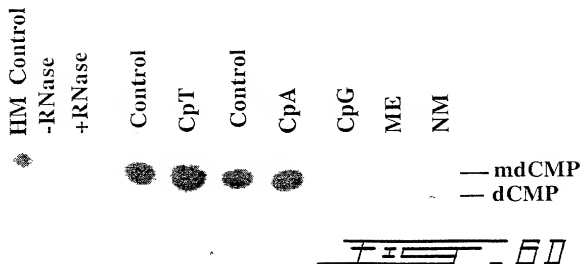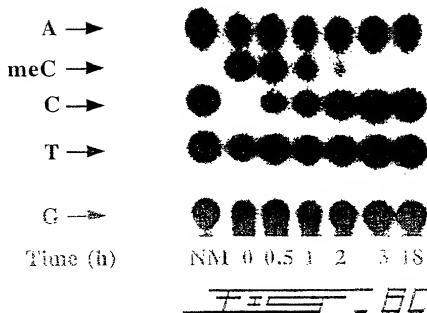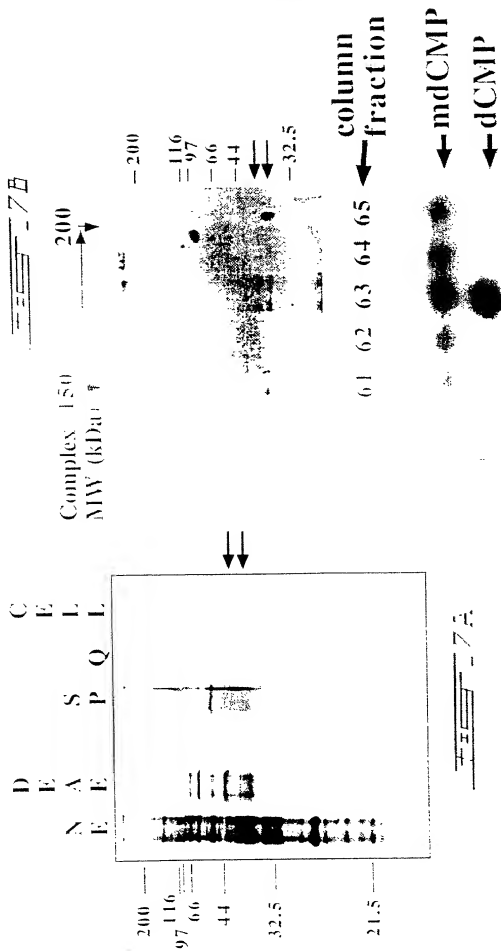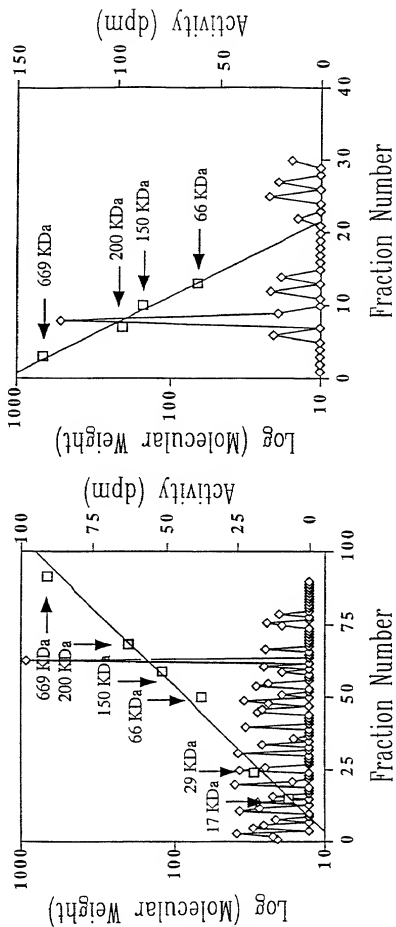
MDCPALPPGW KKEEVIRKSG LSAGKSDVYY FSPSGKKFRS   40
KPQLARYLGN TVDLSSFDFR TGKMMPSKLQ KNKQRLRNDP   80
LNQNKGKPDL NTTLPIRQTA SIFKQPVTKV TNHPSNKVKS   120
DPQRMNEQPR QLFWEKRLQG LSASDVTEQI IKTMELPKGL   160
QGVGPGSNDE TLLSAVASAL HTSSAPITGQ VSAAVEKNPA   200
VWLNTSQPLC KAFIVTDEDI RKQEERVQQV RKILEDALMA   240
DILSRAADTE EMDIEMDSGD EA   262

~~~ homology to methylated DNA binding domain

▨▨ homology to coiled coil domain

FIG. 8B

brain heart kidney stomach testis placenta

9.0

6.0
5.0
4.0

3.0
2.5

2.0
1.5

1.0

0.5

dMTase

β−actin

Human DNA demethylase cDNA-dMTase1 and predicted amino acid sequence

```
5'ggggccgtgg  cccgagaag   gcggagacaa  gatggccgcc  catagcgctt  ggaggaccta
  agaggcggtg  gccggggcca  cgccccgggc  aggagggccg  ctctgtgcgc  gccgctcta
  tgatgcttgc  gcgcgtcccc  cgcgcgccgc  gctgcgggcg  ggcgcgggtct ccgggattcc
  aagggctcgg  ttacggaaga  agcgcagcgc  cggctgggga  ggggctgga   tgcgcgcgca
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  cccggtgagc  ggcgtgcgca  ggaaggcgc   tcggggcggc  ggccgtggcc  ggggcggtg
  gaagcaggcg  ggcgggggcg  gccggggccg  cgggccggc   ccgagtggcg  gcagcggcct
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  ggagccggtc  ccttttcccgt cgggggacgc  gggggccggg  cccagggac   aggaggaagt
  ggagagcggg  aagaggatgg  attgcccggc  cctccccccc  ggatggaaga  aggaggaagt
  gatccgaaaa  tctgggctaa  gtgctggcaa  gagcgatgtc  tactacttca  gtccaagtgg
  taagaagttc  agaagcaagc  ctcagttggc  aaggtacctg  ggaaatactg  ttgatctcag
  cagtttgac   ttcagaactg  gaaagatgat  gctagtaaa   ttacagaaga  acaaacagag
  actgcgaaac  gatcctctca  atcaaaataa  ggtaaaacca  gacttgaata  caacattgcc
  aattagacaa  acagcatcaa  ttttcaaaca  accggtaacc  aaagtcacaa  atcatcctag
```

```
taataaagtg aaatcagacc cacaacgaat gaatgaacag ccacgtcagc ttttctggga
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actacccaaa gtcttcaag  gagttggtcc aggtagcaat gatgagaccc ttttatctgc
tgttgccagt gctttgcaca caagctctgc gccaatcaca gggcaagtct cogctgctgt
ggaaaagaac cctgctgttt ggcttaacac atctcaaccc ctctgcaaag ctttattgt
cacagatgaa gacatcagga aacaggaaga gcgagtacag caagtacgca agaaattgga
agaagcactg atggcagaca tcttgtcgcg agctgctgat acagaagaga tggatattga
aatggacagt ggagatgaag cctaagaata tgatcaggta actttcgacc gactttcccc
aagrgaaaat tcctagaaat tgaacaaaaa tgtttccact ggctttgtcc tgtaagaaaa
aaaatgtacc cgagcacata gagctttta  atagcactaa ccaatgcctt tttagatgta
tttttgatgt atatatctat tattcaaaaa atcatgttta tttggagtcc taggacttaa
aattagtctt ttgtaatatc aagcaggacc ctaagatgaa gctgagcttt tgatgccagg
tgcaatctac tggaaatgta gcacttacgt aaaacatttg ttctcccccac agttttaata
agaacagatc aggaattcta aataaatttc ccagttaaag attattgtga cttcactgta
tataaacata tttttatact ttattgaaag gggacacctg tacattcttc catcatcact
gtaaagacaa ataaatgatt atattcacaa aaaaaaaaaa aaaaaa          3'
```

SEQ ID NO:1

FIG. 9B

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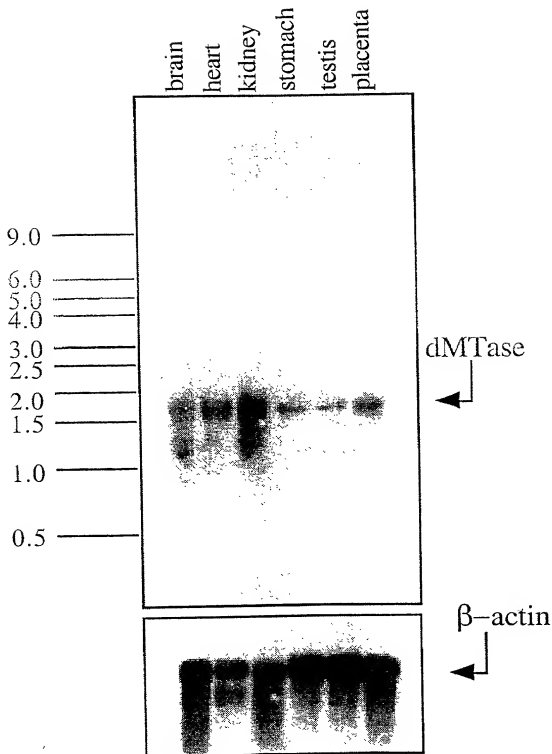REGARGGGRGRGRWKQAGRGGVCGRGRGRGRGRGRGRGRGRPPSG
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VDLSSFDFRTGKMMPSKLQKNKQRLRNDPLNQNKGKPDLNTTLPIRQTAS
IFKQPVTKVTNHPSNKVKSDPQRMNEQPRQLFWEKRLQGLSASDVTEQII
KTMELPKGLQGVGPGSNDETLLSAVASALHTSSAPITGQVSAAVEKNPAV
WLNTSQPLCKAFIVTDEDIRKQEERVQQVRKKLEEALMADILSRAADTEE
MDIEMDSGDEA

SEQ ID NO:2

FIG. 78

FIG-96

Human DNA demethylase homologue-dMTase2 and predicted amino acid sequence

```
5' agcgggccga  ggagccgggc  gcaatggagc  ggaagaggtg  ggagtgccg   gcgctcccgc
   aggctggga   gaggaagaa   gtgcccagaa  ggtcgggct   gtcggccggc  cacagggatg
   tctttacta   tagcccgagc  gggaagaagt  tccgcagcaa  gccgcagctg  gcgcgctacc
   tgggcggctc  catggaccctg agcaccttcg  acttccgcac  gggcaagatg  ctgatgagca
   agatgaacaa  gagccgccag  cgcgtgcgct  acgactcctc  caaccaggtc  aagggcaagc
   ccgacctgaa  cacggcgctg  ccgtgcgcc   agacggcgtc  catcttcaag  cagccggtga
   ccaagattac  caaccacccc  agcaacaagg  tcaagagcga  cccgcagaag  gcggtggacc
   agccgcgcca  gctcttctgg  gagaagaagc  tgagcggcct  gaacgccttc  gacattgctg
   aggagctggt  caagaccatg  gacctcccca  aggccctgca  ggggtggga   cctggctgca
   cggatgagac  gctgctgtcg  gccatcgcca  gcgccctgca  cactagcacc  atgcccatca
   cggagacagct ctcggccgc   gtggagaaga  acccggcgt   atggctcaac  accacgcagc
   ccctgtgcaa  agccttcatg  gtgaccgacg  aggacatcag  gaagcaggaa  gagctggtgc
   agcaggtgcg  gaagcggctg  gaggaggcgc  tgatggccga  catgctggcg  cacgtggagg
   agctggcccg  tgacggggag  gcgccgctgg  acaaggcctg  cgctgaggac  gacgacgagg
   aagacgagga  ggaggaggag  gaggagcccg  accccgaccc  ggagatggag  cacgtctagg
   gcagaggccc  tgccgagagc  ccgtgctgcc  tgctggagcc  gcctgcagac  cggtcctcg
   gccccacgtg  aaccaggctc  gcggcgaag   ccaggccttg  gagacaccca  ggaggaaggc
   cgtgctcctg  gctcctcct   cggccgtcc   ccacttccg   ggcctcggg   gcacacagct
   gggctgccc   ccaccgaaa   gaccctccac  gctcgtcctc  tacagagtcc  ggcttcggga
   agtgccgggt  gctcctgggc  cctgctggc   tccctacgac  cttgggctc   gaggccgagt
```

```
cctcccatg  ccgtgtcc   cagctcctg  agactggaga  gcagccagca  ggtgccggc
agctcggcgc cacggcttgc tgacagctgg gagggtttct  cgttctggag  gcgtagtttt
gaaactcaca tcacccactg tgcaggtga  ggacggact   ctggtctgct  gtggggggca
tgcaggacgg cgccactctc tgccctgcca tgcggctggt  ggtgccacag  agcctcaccg
tgcctgagtg gcgtgccag  ggaggcgct  ctccttcagt  aaatgtaaca  cagtcgaggc
acgtcatcgg gcagcttcc  ctgtgtgcca acgccagcct  tcgcttctga  aaaccaaact
ccagccgctg ccagtcgga  cttggtcgc  cggcggcgcc  agaatgctcc  actgccagcc
ggcccccctg cctcggtttc ccttctgttt agtggcgaca  caggcaccca  gcttgggt
ggtgctgacg ctcccagggg tgccaggagc cactgggaca  gggtgaggct  cccagacgct
cctcgaggtg cccagctctc caggagctt  ctggcccaag  gcgttcttga  gggatctgct
ccttaacccc ccagtgcctt ggcgagggca ggttccaagc  cacagacgcc  tgccccgagt
ggactttgcg gccagtccct gggtgccttc ctgggccctg  cttgcccagt  gagggttcct
aacgggtggg ttcawtggcc tggcccvagc gagcccccac  ctgcattgac  cttaggccca
tagagaggc  ctgtcccggt gctgcccag  ccaaggatct  ggtcgctgcc  ccaggggac
tgatgggcaa gagtcgccgc tgtggctgga ctgtgaccat  ccctgatggg  gctgacccgc
gggagctgag gaagcgccgc tccaccgtct gccctccaag  gaccgcatg   gaggcagtgg
gctggcagct tcctgctgct cctgtcaga  gtcaaagcac  aaatcctcag  gacgggctca
aggccaggg  cagccgaggg aagctccagg tgggaccac   gtcttcctga  ggttggtgcc
cactggctgg gacccttgc  agtgggtgg  cctccctct   gtctgcctgg  tggaggggagc
cgtgggcgtg gggacgtgac tgaataaagc caccatgggt  ggatgtgctt  gg 3'

                                              SEQ ID NO:3
```

FIG. 9E

MERKRWECPALPQGWEREEVPRRSGLSAGHRDVFYSPSGKKFRSKPQLA
RYLGGSMDLSTFDFRTGKMLMSKMNKSRQRVRYDSSNQVKGKPDLNTALP
VRQTASIFKQPVTKITNHPSNKVKSDPQKAVDQPRQLFWEKKLSGLNAFD
IAEELVKTMDLPKGLQGVGPGCTDETLLSAIASALHTSTMPITGQLSAAV
EKNPGVWLNTTQPLCKAFMVTDEDIRKQEELVQQVRKRLEEALMADMLAH
VEELARDGEAPLDKACAEDDDEEDEEEEEEPDPDPEMEHV

SEQ ID NO:4

FIG. 9F

Lipman-Pearson Protein Alignment
Ktuple: 2; Gap Penalty: 4; Gap Length Penalty: 12

		Similarity	Gap	Gap	Consensus
Seq1(1>411)	Seq2(1>291)	Index	Number	Length	Length
human dMTase1 protein	human dMTase2 protein	76.0	0	0	250
(148>253)	(4>253)	76.0	0	0	250

```
                   v150        v160        v170        v180        v190        v200        v210
KRMDCPALPPGWKKEEVIRKSGLSAGKSDVYYFSPSGKKFRSKPQLARYLGNIVDLSSFDFRIGKMMPSK
KR :CPALP.GW.:EEV R:SGLSAG..DV:Y:SPSGKKFRSKPQLARYLG.::DLS:FDFRIGKM: SK
KRWDCPALPQGWEREEVPRRSGLSAGHRDVFYYSPSGKKFRSKPQLARYLGGSMDLSTFDFRIGKMLMSK
    ^10         ^20         ^30         ^40         ^50         ^60         ^70

                   v220        v230        v240        v250        v260        v270        v280
LQRNKQRLRNDFLNQNKGKPDINTTLPTRQTASLFKQPVIKVINHPSNKVKSDPQRMNEQPRQLFWEKRL
::K::QR:R D: NQ KGKPDINT:LP:RQTASLFKQPVIK:TNHPSNKVKSDPQ: :QPRQLFWEK:L
MNKSRQRVRYDSSNQVKGKPDINTALPVRQTASLFKQPVIKITNHPSNKVKSDPQKAVDQPRQLFWEKKL
    ^80         ^90         ^100        ^110        ^120        ^130        ^140
```

FIG. - 96

```
           v290       v300       v310       v320       v330       v340       v350
QGLSASDVIEQIKTMELPKGLQGVGPGSNDEILSAVASALHISSAPIIGQVSAAVEKNPAWLNISQP
GL:A D::E:::KIM::LPKGLQGVGPG..DEILLSA:ASALHIS: PIITGQ:SAAVEKNP:VWLNT:QP
SGLNAFDTAFELVKIMDLPKGLQGVGPGCTDEILSAIASALHTSIMPITGQLSAAVEKNPGVWLNITQP
           ^150       ^160       ^170       ^180       ^190       ^200       ^210

           v360       v370       v380       v390
LCKAFIVIDEDIRKQEERVQQVRKKLEEEAIMADILSRAAD
LCKAF:VIDEDIRKQEE VQQVRK:LEEEAIMAD:L::...:
LCKAFMVIDEDIRKQEEELVQQVRKRLEEEAIMADMLAHVEE
           ^220       ^230       ^240       ^250
```

FIG. 9H

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Mouse DNA demethylase-dMTase1 and predicted amino acid sequence

```
5' ccgctctgcg ggcggggcgg gtctccggga ttccaagggc tcggttacgg aagagcgca
   gagccggctg ggagggggc  tggatgcgcg cgcaccaggg gggaggccgc tgctgcccgg
   agcaggagga ggggagagc  gcggcgggcg gcagcggcgc tgccggcgac tccgccatag
   agcagggggg ccagggcagc gcgctcgctc cgtccccggt gagcggcgtg cgcaggggaag
   gcgctcgggg cggcggccgt ggcgggggc  ggtggaagca ggcggccgg  gggggcggcg
   tctgtggccg tggccgtggc cgtggccgg  gtcggggccg tggccggggc cgggggcggg
   gccgcggccg tccccagagt ggcggcagcg gccttggcgg cgacggcgg  gggggcgcgg
   gcggctgcgg cgtcggcagc ggtggcggcg tcgccccccg gcgggatcct gtccctttcc
   cgtcggggag ctcggggccg gggcccaggg gaccccgggc cacggagagc gggaagagga
   tggactgccc ggccctcccc ccggatgga  agaaggagga agtgatccga aaatcagggc
   tcagtgctgg caagagcgat gtctactact tcagtccaag tggtaagaag ttcagaagta
   aacctcagct ggcaagatac ctgggaaatg ctgttgacct tagcagtttt gacttcagga
   ccggcaagat gatgcctagt aaattacaga gaacaagca  gagactccgg aatgacccc
   tcaatcagaa caagggtaaa ccagacctga acacaacatt gccaattaga caaactgcat
   caatttcaa  gcaaccagta accaaattca cgaaccaccc gagcaataag gtgaagtcag
   accccagcg  gatgaatgaa caaccacgtc agctttctg  ggagaagagg ctacaaggac
   ttagcgcatc agatgtaaca gaacaaatta taaaaaccat ggagctacct aaaggtcttc
   aaggagtcgg tccaggtagc aatgacgaga ccctctgtc  tgctgtggcc agtgctttac
```

```
acacaagctc tgcgcccatc acaggacaag tctctgctgc cgtggaaaag aaccctgctg
tttggcttaa cacatcctcaa aagctttcat tgttacagat gaagacatta
ggaaacagga agagcgagtc caacaagtac gcaagaaact ggaggaggca ctgatggccg
acatcctgtc ccgggctgcg gacacggagg aagtagacat tgacatggac agtggagatg
aggcgtaaga atatgatcag gtaactttcg actgaccttc cccaagagca aattgctaga
aacagaatta aaacatttcc actgggtttc gcctgtaaga aaaagtgtac ctgagcacat
agctttttaa tagcactaac caatgccttt ttagatgtat tttgatgtat tatatctatt
attccaaatg atgttatttt tgaatcctag gacttaaaat gagtctttta taatagcaag
cagggccctt ccggtgcagt gcagctttga ggccaggtgc agtctactgg aaaggtagca
cttacgtgaa atatttgttt ccccacacgt tttaatataa acagatcagg agtaccaaat
aagtttccca attaaagatt attatacttc actgtatata aacagatttt tatactttat
tgaaagaaga tacctgtaca ttcttccatc atcactgtaa agacaaataa atgactatat
tcac 3'
```

SEQ ID NO:5

FIG - 9J

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SEQ ID NO:6

MRAHPGGGRCCPEQEEGESAAGGSGAGDSAIEQGGGQGSALAPSPVSGVR
REGARGGGRGRGRWKQAARGGGVCGRGRGRGRGRGRGRGRGRGRGRPQSG
GSGLGGDGGGGAGGCGVGSGGGVAPRRDPVPFPSGSSGPGPRGPRATESG
KRMDCPALPPGWKKEEVIRKSGLSAGKSDVYYFSPSGKKFRSKPQLARYL
GNAVDLSSFDFRTGKMPSKLQKNKQRLRNDPLNQNKGKPDLNTTLPIRQ
TASIFKQPVTKFTNHPSNKVKSDPQRMNEQPRQLFWEKRLQGLSASDVTE
QIIKTMELPKGLQGVGPGSNDETLLSAVASALHTSSAPITGQVSAAVEKN
PAVWLNTSQPLCKAFIVTDEDIRKQEERVQQVRKKLEEALMADILSRAAD
TEEVDIDMDSGDEA

Mouse DNA demethylase-dMTase2 and predicted amino acid sequence

FIG - 76

```
5'cacgcgcggg cgggtgggcg gagcggcccc cctagcgggg gctgtgaagc gcggggaggg
  ggccgagcgg gtgcgcaagc cggcgcgcgc ccggctgggg gcggaggcgg gaggcccgtg
  ggacagaaca gctgcggcga gtggcggcgg cggagggagc cgaatcggcg acgagcccgg
  gggtcgcaac ttgcagaagc ggcggcggcg gcggcatcgg ccacggcggg cggaaaagcc
  gggcgcaat ggagcggaag agtgggagt gccggcgct cccgcagggc tgggaaaggg
  aagaagtgcc caggaggtcg gggctgtcgg ccggccacag ggatgtcttt tactatagcc
  ccagcgggaa gaagttccgc agcaagccac aactggcacg ttacctgggc ggatccatgg
  acctcagcac cttcgacttc cgcaccggaa agatgttgat gaacaagatg aataagagtc
  gccagcgtgt gcgctatgat tcttccaacc aggtcaaggg caagcctgac ctgaacaccg
  cgctgcctgt acggcagact gcatccatct tcaagcaacc ggtgaccaag atcaccaacc
  acccagcaa caaggtcaag agcgacccgc agaaggcagt ggaccagccg aggcagcttt
  tctgggagaa gaagctaagt ggattgagtg cctttgacat tgcagaagaa ctggtcagga
  ccatggactt gcccaagggc ctgcagggag tgggccctgg ctgtacagat gagacgctgc
  tgtcagccat tgcgagtgct ctacacacca gcaccctgcc cattacaggc cagctctctg
  cagccgtgga gaagaaccct ggtgtgtggc tgaacactgc acagccactg tgcaaagcct
  tcatggtgac agatgacgac atcaggaagc aggaggagct ggtacagcag gtacggaagc
  gcctggagga ggcactgatg gccgacatgc tagctcatgt ggaggagctt gcccgagacg
  gggaggcacc actggacaag gcctgtgcag aggaggaaga ggaggaggag ggaggaggag
```

```
aagagccgga  gccagagcga  gtgtagcaca  ggtgccctgc  ccaagtctgg  gctgcagact
gccttcagcc  ttgcctggac  caggtagggg  ccagacctgt  aggaggcagc  cgtccacctc
cttccaaag   cctcctgctt  ccaggtctca  gtgcagggag  ccctgtgga   ccttgaactc
acttgtccct  gcgctgcctg  gcaggaagcc  ccacactgaa  agcagatgag  cagtgaccca
actgagaggc  cacctggaca  cagtcacctc  cctgcctcct  tatcatagga  caaggccttg
cttggcaccg  aggagctggg  agccgtgttg  ggtgctggag  gaagtttctg  gaaacacacc
tggctatgcc  caccttatgt  ccctaaggct  attacaggcc  agggtttgga  ctgctccggc
ccacagggct  gcccagcctc  cccacactga  gggtcagcag  cccaccagga  agtcactttc
cttcaataaa  ctgatggtag  gaacttgtg   3'
```

SEQ ID NO:7

FIG. 9M

MERKRWECPALPQGWEREEVPRRSGLSAGHRDVFYYSPSGKKFRSKPQLA
RYLGGSMDLSTFDFRTGKMLMNKMNKSRQRVRYDSSNQVKGKPDLNTALP
VRQTASIFKQPVTKITNHPSNKVKSDPQKAVDQPRQLFWEKKLSGLSAFD
IAEELVRTMDLPKGLQGVGPGCTDETLLSAIASALHTSTLPITGQLSAAV
EKNPGVWLNTAQPLCKAFMVTDDIRKQEELVQQVRKRLEEALMADMLAH
VEELARDGEAPLDKACAEEEEEEEEEEEPEPERV

SEQ ID NO:8

FIG - 9N

Lipman-Pearson Protein Alignment
Ktuple: 2; Gap Penalty: 4; Gap Length Penalty: 12

Seq1(1>414)	Seq2(1>285)		Similarity Index	Gap Number	Gap Length	Gap Length	Consensus Length
mouse dMTase1 protein	mouse dMTase2 protein						
(151>400)	(4>253)		75.2	0	0		250
(151>400)	(4>253)		75.2	0	0		250

```
              v160  v170  v180  v190  v200  v210  v220
               ^10   ^20   ^30   ^40   ^50   ^60   ^70

KRMDCPALPPGMKKEEVIRKSGLSAGKSDVYFSPSGKKFRSKPQLARYLGNAVDLSSFDFRTGKMMPSK
KR :CPALP.GW.:EEV R:SGLSAG..IV:Y:SPSGKKFRSKPQLARYLG.::DLS:FDFRTGKM: :K
KRWECPALPQGWEREEVPRRSGLSAGHRDVFYYSPSGKKFRSKPQLARYLGGSMDLSTFDFRTGKMLMNK

              v230  v240  v250  v260  v270  v280  v290
               ^80   ^90  ^100  ^110  ^120  ^130  ^140

LQRNKQRLRNDPLNQNKGKPDINITTLPIRQTASIFKQPVTKFINHPSNKVKSDPQRMNEQPRQLFWEKRL
::K:.:QR:R D: NQ KGKPDINT:LP:RQTASIFKQPVTK.:INHPSNKVKSDPQ:  :QPRQLFWEK:L
MNKSRQRVRYDSSNQVKGKPDINIALPVRQTASIFKQPVTKTINHPSNKVKSDFQKAVDQPRQLFWEKKL
```

FIG - 90

```
        v300      v310      v320      v330      v340      v350      v360
QGLSASDVTEQLIKTMELPKGLQGVGPGSNDETLLSAVASALHTSSAPITGVSAAVEKNPAVWLNTSQP
GLSA D::E::::TM:LPKGLQGVGPG..DETLLSA:ASALHTS: PITGQ:SAAVEKNP:VWLNT:QP
SGLSAFDIAEELVRIMDLPKGLQGVGPGCTDETLLSAIASALHTSTLPITGQLSAAVEKNPGVWLNTAQP
      ^150      ^160      ^170      ^180      ^190      ^200      ^210

            v370      v380      v390      v400
LCKAFIVTDEDIRKQEERVQQVRKKLEFEALMADILSRAAD
LCKAF:VID:DIRKQEE VQQVRK:LEFEALMAD:L:::::
LCKAFMVIDDIRKQEELVQQVRKRLEFEALMADMLAHVEE
      ^220      ^230      ^240      ^250
```


FIG. 9P

FIG.-10A

Fig. 10B

Fig. 11A

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FIG. 11E

me SK + *Hpa* II

dMTase⌐ ⌐Buffer L
dMTase+
Protease K

0.71
0.49,0.40
0.32
0.24
0.15

FIG. 11D

dMTase
NM
ME

mdCMP
dCMP

pBluescript
SK+

← substrate →

FIG. 11B

dMTase
empty vector

250—
148—
60—
42—
30—

FIG. 11C

dMTase + Protease K
misframe
dMTase
ME
NM

(mdCpdG)n

$FIG. 11F$

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FIG. 12A

FIG. 12B

FIG. 13A

FIG. 13B

FIG. 13C

brain heart kidney stomach testis liver spleen Y1

dMTase

← ~1.5 kb

18s

dMTase / 18s

0.020
0.016
0.012
0.008
0.004
0.000

brain heart kidney stomach testis liver spleen Y1

Fig. 130

FIG. 14A

FIG. 14B

FIG. 14C

Antisense

Sense

pRetro-ON dMTase

FIG. 15

FIG. 16

50/50

FIG. 17

COMBINED DECLARATION FOR UTILITY OR DESIGN
PATENT APPLICATION (37 CFR 1.63) AND POWER OF ATTORNEY

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/CA98/01059	11/12/1998	

☐ Additional U.S. or PCT International application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent Trademark Office connected therewith:

➤ ☐ Customer Number

OR ☒ Registered practitioner(s) name/registration number listed below

Place Customer Number Bar Code Label Here

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Donald D. Evenson	26,160	Gary R. Edwards	31,824
Robert Mitchell	25,007	Guy Houle	24,971
Kevin P. Murphy	26,674	Robert Carrier	30,726
Michel J. Sofia	37,017	François Nadeau	37,570
France Côté	37,037		

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Moshe	SZYF

Inventor's Signature		Date	28. 6. 2000
Residence: City	Côte St-Luc	State	Québec	Country	Canada	Citizenship	Canadian
Post Office Address	7522 Wavell						

City	Côte St-Luc	Province or State	Québec	Postal Code Or Zip	H4W 2L7	Country	Canada

☒ Additional inventors are being named on the   supplemental Additional Inventor(s) PTO/SB/02A attached hereto.

DECLARATION	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 3 of 3

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

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---

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])   Family Name or Surname

Inventor's Signature _____   Date _____

Residence:
City _____ State _____ Country _____ Citizenship _____

Post Office Address _____

City _____ Province or State _____ Postal Code Or Zip _____ Country _____

☐ Additional inventors are being named on the   supplemental Additional Inventor(s) PTO/SB/02A attached hereto.

SEQUENCE LISTING

<110> McGILL UNIVERSITY
      SZYF, Moshe
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      RAMCHANDANI, Shyam

<120> DNA DEMETHYLASE, THERAPEUTIC AND
  DIAGNOSTIC USES THEREOF

<130> 1770-183"PCT" FC/ld

<150> CA 2,220,805
<151> 1997-11-12

<150> CA 2,230,991
<151> 1998-05-11

<160> 10

<170> FastSEQ for Windows Version 3.0

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<213> Unknown

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agcaggagga gggggagagc gcggcgggcg gcagcggcgc tggcggcgac tccgccatag   180
agcagggggg ccagggcagc gcgctcgctc cgtccccggt gagcggcgtg cgcagggaag   240
gcgctcgggg cggcggccgt ggccgggggc ggtggaagca ggcggcccgg ggcggcggcg   300
tctgtggccg tggccgtggc cgtggccggg gtcggggccg gtgccgggc cgggccgggg   360
gccgcggccg tccccagagt ggcggcagcg gccttggcgg cgacggcggc ggcggcgcgg   420
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cgtcggggag ctcggggccg gggcccaggg gaccccgggc cacggagagc gggaagagga   540
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            20                  25                  30
Glu Gln Gly Gly Gln Gly Ser Ala Leu Ala Pro Ser Pro Val Ser Gly
        35                  40                  45
Val Arg Arg Glu Gly Ala Arg Gly Gly Gly Arg Gly Arg Gly Arg Trp
    50                  55                  60
Lys Gln Ala Gly Arg Gly Gly Gly Val Cys Gly Arg Gly Arg Gly Arg
65                  70                  75                  80
Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg
            85                  90                  95
Pro Pro Ser Gly Gly Ser Gly Leu Gly Gly Asp Gly Gly Gly Cys Gly
            100                 105                 110
Gly Gly Gly Ser Gly Gly Gly Gly Ala Pro Arg Arg Glu Pro Val Pro
        115                 120                 125
Phe Pro Ser Gly Ser Ala Gly Pro Gly Pro Arg Gly Pro Arg Ala Thr
    130                 135                 140
Glu Ser Gly Lys Arg Met Asp Cys Pro Ala Leu Pro Pro Gly Trp Lys
145                 150                 155                 160
Lys Glu Glu Val Ile Arg Lys Ser Gly Leu Ser Ala Gly Lys Ser Asp
                165                 170                 175
Val Tyr Tyr Phe Ser Pro Ser Gly Lys Lys Phe Arg Ser Lys Pro Gln
            180                 185                 190
Leu Ala Arg Tyr Leu Gly Asn Thr Val Asp Leu Ser Ser Phe Asp Phe
        195                 200                 205
Arg Thr Gly Lys Met Met Pro Ser Lys Leu Gln Lys Asn Lys Gln Arg
    210                 215                 220
Leu Arg Asn Asp Pro Leu Asn Gln Asn Lys Gly Lys Pro Asp Leu Asn
225                 230                 235                 240
Thr Thr Leu Pro Ile Arg Gln Thr Ala Ser Ile Phe Lys Gln Pro Val
                245                 250                 255
Thr Lys Val Thr Asn His Pro Ser Asn Lys Val Lys Ser Asp Pro Gln
            260                 265                 270
Arg Met Asn Glu Gln Pro Arg Gln Leu Phe Trp Glu Lys Arg Leu Gln
        275                 280                 285
Gly Leu Ser Ala Ser Asp Val Thr Glu Gln Ile Ile Lys Thr Met Glu
    290                 295                 300
Leu Pro Lys Gly Leu Gln Gly Val Gly Pro Gly Ser Asn Asp Glu Thr
305                 310                 315                 320
Leu Leu Ser Ala Val Ala Ser Ala Leu His Thr Ser Ser Ala Pro Ile
                325                 330                 335
Thr Gly Gln Val Ser Ala Ala Val Glu Lys Asn Pro Ala Val Trp Leu
            340                 345                 350
Asn Thr Ser Gln Pro Leu Cys Lys Ala Phe Ile Val Thr Asp Glu Asp
            355                 360                 365
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Ile Arg Lys Gln Glu Glu Arg Val Gln Gln Val Arg Lys Lys Leu Glu
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Glu Ala Leu Met Ala Asp Ile Leu Ser Arg Ala Ala Asp Thr Glu Glu
385                 390                 395                 400
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tggctatgcc cacctttatgt ccctaaggct attacaggcc agggtttgga ctgctccggc   1500
ccacagggct gcccagcctc cccacactga gggtcagcag cccaccagga agtcactttc   1560
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        <211> 291
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                20                  25                  30
Val Phe Tyr Tyr Ser Pro Ser Gly Lys Lys Phe Arg Ser Lys Pro Gln
            35                  40                  45
Leu Ala Arg Tyr Leu Gly Gly Ser Met Asp Leu Ser Thr Phe Asp Phe
    50                  55                  60
Arg Thr Gly Lys Met Leu Met Ser Lys Met Asn Lys Ser Arg Gln Arg
65                  70                  75                  80
```

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Val Arg Tyr Asp Ser Ser Asn Gln Val Lys Gly Lys Pro Asp Leu Asn
                85                  90                  95
Thr Ala Leu Pro Val Arg Gln Thr Ala Ser Ile Phe Lys Gln Pro Val
            100                 105                 110
Thr Lys Ile Thr Asn His Pro Ser Asn Lys Val Lys Ser Asp Pro Gln
        115                 120                 125
Lys Ala Val Asp Gln Pro Arg Gln Leu Phe Trp Glu Lys Lys Leu Ser
    130                 135                 140
Gly Leu Asn Ala Phe Asp Ile Ala Glu Glu Leu Val Lys Thr Met Asp
145                 150                 155                 160
Leu Pro Lys Gly Leu Gln Gly Val Gly Pro Gly Cys Thr Asp Glu Thr
            165                 170                 175
Leu Leu Ser Ala Ile Ala Ser Ala Leu His Thr Ser Thr Met Pro Ile
        180                 185                 190
Thr Gly Gln Leu Ser Ala Ala Val Glu Lys Asn Pro Gly Val Trp Leu
    195                 200                 205
Asn Thr Thr Gln Pro Leu Cys Lys Ala Phe Met Val Thr Asp Glu Asp
    210                 215                 220
Ile Arg Lys Gln Glu Glu Leu Val Gln Gln Val Arg Lys Arg Leu Glu
225                 230                 235                 240
Glu Ala Leu Met Ala Asp Met Leu Ala His Val Glu Glu Leu Ala Arg
            245                 250                 255
Asp Gly Glu Ala Pro Leu Asp Lys Ala Cys Ala Glu Asp Asp Asp Glu
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Glu His Val
    290
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cccggtgagc ggcgtgcgca gggaaggcgc tcggggcggc ggccggtggcc tcgccccgtc    360
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            20                  25                  30
Glu Gln Gly Gly Gln Gly Ser Ala Leu Ala Pro Ser Pro Val Ser Gly
        35                  40                  45
Val Arg Arg Glu Gly Ala Arg Gly Gly Gly Arg Gly Arg Gly Arg Trp
    50                  55                  60
Lys Gln Ala Ala Arg Gly Gly Gly Val Cys Gly Arg Gly Arg Gly Arg
65              70                  75                  80
Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg Gly Arg
            85                  90                  95
Pro Gln Ser Gly Gly Ser Gly Leu Gly Gly Asp Gly Gly Gly Gly Ala
        100                 105                 110
Gly Gly Cys Gly Val Gly Ser Gly Gly Gly Val Ala Pro Arg Arg Asp
    115                 120                 125
Pro Val Pro Phe Pro Ser Gly Ser Ser Gly Pro Gly Pro Arg Gly Pro
    130                 135                 140
Arg Ala Thr Glu Ser Gly Lys Arg Met Asp Cys Pro Ala Leu Pro Pro
145                 150                 155                 160
Gly Trp Lys Lys Glu Glu Val Ile Arg Lys Ser Gly Leu Ser Ala Gly
                165                 170                 175
Lys Ser Asp Val Tyr Tyr Phe Ser Pro Ser Gly Lys Lys Phe Arg Ser
            180                 185                 190
Lys Pro Gln Leu Ala Arg Tyr Leu Gly Asn Ala Val Asp Leu Ser Ser
        195                 200                 205
Phe Asp Phe Arg Thr Gly Lys Met Met Pro Ser Lys Leu Gln Lys Asn
    210                 215                 220
Lys Gln Arg Leu Arg Asn Asp Pro Leu Asn Gln Asn Lys Gly Lys Pro
225                 230                 235                 240
Asp Leu Asn Thr Thr Leu Pro Ile Arg Gln Thr Ala Ser Ile Phe Lys
                245                 250                 255
Gln Pro Val Thr Lys Phe Thr Asn His Pro Ser Asn Lys Val Lys Ser
            260                 265                 270
Asp Pro Gln Arg Met Asn Glu Gln Pro Arg Gln Leu Phe Trp Glu Lys
        275                 280                 285
Arg Leu Gln Gly Leu Ser Ala Ser Asp Val Thr Glu Gln Ile Ile Lys
    290                 295                 300
```

```
Thr Met Glu Leu Pro Lys Gly Leu Gln Gly Val Gly Pro Gly Ser Asn
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Asp Glu Thr Leu Leu Ser Ala Val Ala Ser Ala Leu His Thr Ser Ser
                325                 330                 335
Ala Pro Ile Thr Gly Gln Val Ser Ala Ala Val Glu Lys Asn Pro Ala
            340                 345                 350
Val Trp Leu Asn Thr Ser Gln Pro Leu Cys Lys Ala Phe Ile Val Thr
        355                 360                 365
Asp Glu Asp Ile Arg Lys Gln Glu Glu Arg Val Gln Gln Val Arg Lys
    370                 375                 380
Lys Leu Glu Glu Ala Leu Met Ala Asp Ile Leu Ser Arg Ala Ala Asp
385                 390                 395                 400
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                405                 410
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            20                  25                  30
Val Phe Tyr Tyr Ser Pro Ser Gly Lys Lys Phe Arg Ser Lys Pro Gln
        35                  40                  45
Leu Ala Arg Tyr Leu Gly Gly Ser Met Asp Leu Ser Thr Phe Asp Phe
    50                  55                  60
Arg Thr Gly Lys Met Leu Met Asn Lys Met Asn Lys Ser Arg Gln Arg
65                  70                  75                  80
Val Arg Tyr Asp Ser Ser Asn Gln Val Lys Gly Lys Pro Asp Leu Asn
                85                  90                  95
Thr Ala Leu Pro Val Arg Gln Thr Ala Ser Ile Phe Lys Gln Pro Val
            100                 105                 110
Thr Lys Ile Thr Asn His Pro Ser Asn Lys Val Lys Ser Asp Pro Gln
        115                 120                 125
Lys Ala Val Asp Gln Pro Arg Gln Leu Phe Trp Glu Lys Lys Leu Ser
    130                 135                 140
Gly Leu Ser Ala Phe Asp Ile Ala Glu Glu Leu Val Arg Thr Met Asp
145                 150                 155                 160
Leu Pro Lys Gly Leu Gln Gly Val Gly Pro Gly Cys Thr Asp Glu Thr
                165                 170                 175
Leu Leu Ser Ala Ile Ala Ser Ala Leu His Thr Ser Thr Leu Pro Ile
            180                 185                 190
Thr Gly Gln Leu Ser Ala Ala Val Glu Lys Asn Pro Gly Val Trp Leu
        195                 200                 205
Asn Thr Ala Gln Pro Leu Cys Lys Ala Phe Met Val Thr Asp Asp Asp
    210                 215                 220
Ile Arg Lys Gln Glu Glu Leu Val Gln Gln Val Arg Lys Arg Leu Glu
225                 230                 235                 240
Glu Ala Leu Met Ala Asp Met Leu Ala His Val Glu Glu Leu Ala Arg
                245                 250                 255
Asp Gly Glu Ala Pro Leu Asp Lys Lys Ala Cys Ala Glu Glu Glu Glu
                260                 265                 270
Glu Glu Glu Glu Glu Glu Glu Pro Glu Pro Glu Arg Val
        275                 280                 285

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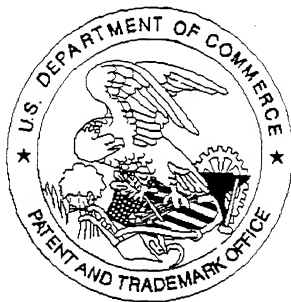
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<213> Unknown

<400> 10
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